The Journal of Biological Chemistry

Volume 2

1906-1907

Reprinted by arrangement with the American Society of Biological Chemists, Inc.

JOHNSON REPRINT CORPORATION
New York, New York

THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

EDITED BY

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Baltimore New York

Assistant Editor, A. N. RICHARDS, New York

VOLUME II.

NEW YORK CITY

1906-07.

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ON BACTERIAL PROCESSES IN THE INTESTINAL TRACT IN SOME CASES OF ADVANCED ANÆMIA, WITH ESPECIAL REFERENCE TO INFECTION WITH B. AEROGENES CAPSULATUS (B. WELCHII).

By C. A. HERTER.

(Received for publication May 10, 1906.)

I have thought it worth while to bring together here a number of observations relating to bacterial processes in the intestinal tracts of persons suffering from severe forms of anæmia, in the belief that they afford a clue to the etiology of many cases of advanced blood disease that are ordinarily described as "primary." Most of the instances which form the basis of this study have given the blood-picture of so-called idiopathic, pernicious anæmia.

The observations relate especially to seventeen cases of anæmia. Of these nine showed those changes in the morphology of the red cells (including the presence of megaloblastic forms) which are regarded as most characteristic of primary pernicious anæmia (Cases I, III, V, VII, VIII, X, XII, XIII, XVII). In four other cases (Cases II, IV, VI, and IX) the diagnosis of pernicious anæmia was made but appears less certain as megaloblasts were not seen. Of the remaining four cases one is an adult from whose history and blood-picture a distinguished consultant leaned to the diagnosis of pernicious anæmia, while another practitioner, upon the same evidence, suspected that he was dealing with an anæmia secondary to gastric carcinoma (XV). Another case is one of severe anæmia which was regarded as secondary, but for which no explanation could be found at autopsy (Case XI). Another case is that of a young child in the care of Dr. Holt, whose history is especially instructive as showing the rapidity of the process of blood regeneration upon the subsidence of a subacute intestinal process to which the anæmia was apparently secondary (Case XVI). The remaining case (XIV) occurred in a child of three

years of age and is probably a splenic anæmia, secondary to some unknown infection. I feel especially indebted to Drs. Spalding, James, Thatcher, and Loomis for placing at my disposal the opportunities for studying the greater number of the patients and for furnishing me with the clinical notes. Dr. Fred. Shattuck of Boston has placed me under obligations for his reports on Case XII.

It is a fact well recognized by those who have studied considerable numbers of patients with pernicious anæmia, that these persons are liable to pronounced derangements of digestion, such as diarrhœa. Among the cases collected here, diarrhœa was a frequent occurrence. It was noted also that even where diarrhœa was absent, the feces were not well formed but were usually of a semi-solid, tarry consistence. And it is worthy of mention that among the many specimens which were sent to the laboratory from persons with pernicious anæmia, since the beginning of the present study, there were very few instances in which the stools have been well formed and of firm consistence throughout or in large part.

Routine microscopical examinations of the stools were made by Dr. W. R. Williams with reference to the presence of meat fibres, vegetable residue, starch, phosphates, free fat, fatty acids, and soaps. Unfortunately the examinations were not made with a sufficiently full knowledge of the nature of the diet in each case to make it safe to form a generalization with reference to the features mentioned. It was noticed that meat fibres and vegetable residue were very abundant in some instances, but the significance of these facts is uncertain, partly because the dietetic conditions are not known in detail, partly because the habits of the patients with respect to mastication were not recorded. An excessive amount of mucus was noted in Cases X, XI, and XII. In Case XII its presence was a regular feature.

Occurrence of Phenol, Indol, and Skatol in the Feces and of their Derivatives in the Urine.

In the present study considerable attention was given to the amounts of the aromatic cleavage products, indol, skatol,

and phenol in the feces and to their derivatives in the urine. These bodies, as is well known, are common accompaniments of putrefactive decomposition in the intestine, and it seemed desirable to learn whether in severe anæmic states, not secondary to obvious pathological processes, there is anything noteworthy as to their production.

It is possible to dismiss in a few words the observations that relate to phenol, or, more correctly, to phenolic substances—phenol and paracresol not being distinguished from each other by the method of determination employed.[1] In the following list of cases the phenol values are expressed in milligrams per hundred grams of feces.

No of Case	Phenol in Mgms. per 100 Gms. Feces
I	2.2; 3.6.
II	14.4; 15.1; 12.8.
V	7.7.
VI	6.6; 6.8.
VII	12.9.
VIII	5.2; 13.1.
X	13.2; 4.1.
XII	10.; 5 7; 12.7; 9.0; 19.5; 11.4; 4.9; 2.2.
XIII	5.7.
XIV	7.8; 5.9.
XV	9.4; 4.5; 11.2
XVII	2 5.

The phenol content of normal feces varies within wide limits and even in the case of presumably normal children and adolescents, with no indications of digestive disorder, may amount to 12 milligrams in 100 grams of fresh material. It is difficult to fix the normal limits for phenol but it is clear that in our cases of anæmia there are none in which the phenol content of the feces was strikingly or persistently above the normal. In Case XII, 19.5 milligrams were found on one occasion but this was evidently an exceptionally high value. The largest quantities we have found were observed in the case of a child of eight years who was slowly convalescing from chronic intestinal indigestion of

[1] Kossel and Penny.

such severity as to retard growth and in whom a moderate degree of anæmia (hæmoglobin, 65 per cent.) had developed. Other children suffering from similar clinical manifestations have given uncommonly high results.[1]

In a number of instances observations were made on the phenol of the urine, but there are only a small number of quantitative determinations. In a large number of cases the distillate from the urine was tested with Millon's reagent, the color reaction being described as slight, moderate, or strong. Many times the urine was tested directly in the cold with Millon's reagent. If the urine contains considerable phenol potassium sulphate, this salt is hydrolyzed under these circumstances and the liberated phenol reacts in a characteristic way.

In Case I the phenol reached 85 milligrams on one occasion, 61 on another, and 27.5 on a third, the results being calculated for twenty-four hours; in Case II it was very strong, on one occasion reaching 82 9; in Case XII it was strong on two occasions (on one of these 56.42 milligrams in twenty-four hours or 36.4 milligrams in 500 c.c. of urine) but was subsequently moderate or slight on three occasions. In Case X eight examinations were made. On three occasions the distillate was negative, twice it gave a slight reaction, once a moderate reaction, and twice a strong one. In Case XIV (in which the diagnosis of pernicious anæmia was extremely doubtful) there were two negative examinations. In Case XV the reaction was very strong, reaching 96 milligrams in twenty-four hours on one occasion and 130 milligrams on another.

Summarizing the observations with respect to phenol it may be said that the phenol values for the feces have in general not been remarkably high, but that the phenol output in the urine has in a majority of instances been greater than normal. The urine in most of the cases exhibited a strong phenol reaction (Cases I, II, IV, VII, and IX) but in Cases X and XII the phenol excretion cannot be said to have ruled high, although these were well defined examples of pernicious anæmia with evidence of

[1] A special report will be made on the bacteriological and chemical conditions in cases of this type.

persistent disorders of intestinal digestion. In two of the five cases in which the phenol reaction from the urine was strong it was exceptionally intense and in all of these cases was sufficiently pronounced to point to excessive intestinal putrefaction. Comparing the phenol reaction in these cases with those obtained from normal children and young adults living under hygienic conditions, the reactions must be regarded as excessive, but they were less marked than in many cases of chronic intestinal indigestion in children and adults, excepting Cases I, II, and XV, in which the values were very high.

The observations relating to the indol of the feces and to the indican of the urine are more numerous than those having to do with phenol. They are also of greater significance for the reason that while the urine is never free from phenolic derivatives, even in health, it is often quite free from indican. Indeed an examination of many urines from children and adolescents in good health, and on a hygienic, mixed diet, makes me believe that anything more than a trace of indican in the urine indicates a departure from ideal conditions of intestinal digestion. The formation of putrefactive products in the intestine and their excretion by the urine is in such small quantities, in the cases to which reference is made, that one cannot avoid the conviction that normal digestion is associated with only slight putrefactive decomposition, despite the sojourn of very large numbers of living bacteria in the large intestine.

The indol content of the feces was measured by means of the naphthaquinone-sodium-monosulphonate method[1] and is in some instances expressed in milligrams. The indican of the urine was not quantitatively determined but was roughly gauged by the Obermeyer reaction. A point to which some attention was given is the relation between the intensity of the indican reaction and the quantity of indol recovered from the feces. No definite proportionality between these was noted; indeed, in a number of instances results were obtained which appeared to negative any close relationship between them.

The results obtained are recorded in the following table:

[1]Herter and Foster, "A Method for the Quantitative Determination of Indol," This Journal, i, p. 257, 1906.

No. of Case	Date	Quantity of Indol in 100 Grams of Feces	Indican Reaction of Urine	Further Data
I	Mar. 23, '06 Mar. 30, '06	Trace "	Very strong Negative	Feces unformed
II	Mar. 21, '06 Mar. 28, '06	Negative "	Strong Trace	Feces formed but soft
III	Mar. 21,'06 Apr. 30, '06	Moderate [0.64 mg.] Negative	Negative Strong; deep purple	Feces soft, containing lumps.
IV	Dec. 18,'05 Jan. 5, '06	Strong Negative	Moderate Negative	Diarrhœal movement
VI	Mar. 17, '06 Mar. 26, '06	3 mg. 3.09 mg.	Strong Negative	Formed and liquid feces
VII	Jan. 18, '06 Mar. 15, '06	Trace - - - -	- - - - Moderate	
VIII	Dec. 30, '05 Jan. 9, '06 Apr. 16, '06	Trace Negative Faint trace	 Slight Negative	
IX	Dec. 4, '05	Very strong 13.0 mg.	Strong	
X	Nov. 28, '05 Nov. 30, '05 Dec. 5, '05 Dec. 8, '05 Dec. 12,'05 Dec. 18,'05 Dec. 28, '05 Feb. 1, '06 Mar. 14, '06 Mar. 27, '06	Very strong 46.9 mg. Negative 11.4 mg. 5.5 mg. Strong trace Negative Slight 0.27 mg. - - - -	Strong Strong Moderate Very strong Strong Negative Slight Strong	Period of diarrhœa.

No. of Case	Date	Quantity of Indol in 100 Grams of Feces	Indican Reaction of Urine	Further Data
XII	Dec. 25,'05	Strong, [7 mg.]	Strong	
	Jan. 2, '06	Trace	Strong	
	Jan. 15,'06	Negative	Strong	
	Feb. 1, '06	Slight	Very strong	
	Mar. 14, '06		Strong	
	Mar. 17, '06	Slight	Negative	
	Mar. 31, '06	Moderately strong		
	Apr. 5, '06	7.7 mg.	Faint trace	
	Apr. 27,'06	28 62 mg.	Slight.	
XIII	Apr. 17,'06	Moderate	Strong	
XIV	Jan. 3, '06	Trace		
	Jan. 10, '06	Faint trace	Negative	
XV	Mar. 30, '06	Negative	Strong; purple color	
	Apr. 4, '06	- - - -	Strong	
	Apr. 30,'06	Negative	Very strong	
XVII	May 5, '06	Faint trace	- - - -	

A consideration of the tabulated data shows that the condi tions were widely varying with respect to the fecal indol and the urinary indican, but that in a majority of the cases there was a distinctly excessive excretion of indican. It sometimes happened, as in Case X, that the indican temporarily disappeared from the urine during a period of diarrhœa and there is little doubt that in such cases the negative reaction was dependent on the rapid passage of the contents of the small intestine through the large intestine. Diarrhœas are frequent among persons with pernicious anæmia and may be responsible for some of the negative results as to indican.

It is noteworthy that in one instance (Case X) in which the feces contained a large quantity of indol and the urine gave a strong indican reaction, there was an abrupt falling off in these when lavage of the colon was begun. Coincidently with this there was a rapid improvement in the condition of the patient

and a strikingly rapid rise in the number of red blood cells and in the quantity of hæmoglobin.

In one case (XIV) the urine was free from indican although diarrhœa was absent. The feces contained very little indol.

A feature of some interest as an evidence of putrefaction is the frequent occurrence of skatol in feces of *persons suffering* from severe anæmia of "idiopathic" origin. Among normal persons on mixed diet, the presence of more than a trace of skatol in the feces appears to be an exceptional occurrence. In Case I skatol was fairly abundant (3 milligrams in 100 grams of feces) although only a trace of indol was present. In Case II skatol was present in traces on two occasions, although indol was absent. In Case III skatol was not found. In Case IV skatol was absent on one occasion when indol was abundant; at another time the skatol reaction was strong while indol was negative. In Case VI indol and skatol were both fairly abundant. In Case VII the skatol reaction was moderately strong but indol was detected only as a trace. The same is true of one sample from Case VIII but in a second specimen from this patient (ten days later) indol was unobtainable and skatol was abundantly present. In Case IX skatol was negative while indol was abundant. In Case X skatol was regularly absent (8 examinations) although indol was frequently present. In Case XII skatol was present only once in five times at which examinations were made. The largest quantity of skatol recovered from any pathological feces was in a case of diabetes on the verge of coma. Here only skatol was obtained, indol being wholly absent.[1] In Case XVII considerable skatol was obtained from the feces but no indol.

It is not yet clear why skatol makes its appearance in the intestinal contents in the course of putrefactive disturbance. Like indol it must be regarded as coming from the tryptophan yielded by proteid, but what the conditions are that determine the formation of one of these substances rather than the other we do not know. In ordinary putrefaction indol is usually

[1] The determination of skatol is made colorometrically by means of the reaction with paradimethylamidobenzaldehyde after distillation from a mixture from which nearly all of any indol present has been removed by precipitation with β-naphthaquinone-sodium-monosulphonate. The details of the method will be soon published.

an early product and skatol, if found at all, a much later one. From the putrefaction of peptone and bouillon media by various micro-organisms, individually and in various combinations, for short periods, I have been unable to obtain skatol. One medium from which I have obtained skatol by putrefaction is one prepared from sheep's brains. This origin of skatol from decomposing brain tissues was known to Nencki many years ago, but no explanation of it has been given. Skatol also appeared in a medium containing salts, tryptophan, alanin, and phenylalanin, after incubation with fecal bacteria. But by far the greatest concentration of skatol in a putrefactive culture was found after two weeks' growth of fecal bacteria in a peptone bouillon medium which had been enriched by the addition of a watery extract prepared from fresh asparagus. In one case the fecal flora from a normal pig were used. Here no indol was formed but a very large quantity of skatol. In another experiment in which the fecal bacteria were derived from a diabetic patient, there was very large production of skatol and slight production of indol.

A consideration of the skatol content of the feces carries one to the question of the significance of the paradimethylamidobenzaldehyde reaction of the urine. It is well known that on the addition of an acid solution of Ehrlich's aldehyde to certain urines a cherry-red reaction is obtained, sometimes in the cold, more often only on the application of heat. The cause of the reaction has been the occasion of some discussion. Erhlich[1] was inclined to attribute it to glycosamin; Neubauer[2] and Bauer[3] refer it to urobilinogen which has passed from the intestine into the urine. I have found that the administration of skatol to men and to monkeys is followed by some intensification of the aldehyde reaction and believe that where skatol is found in fair abundance in the intestine it may contribute to the reaction. It cannot, however, be claimed that in the group of anæmias which is under notice here, there was a close relation between the occurrence of skatol in the intestine and the capacity of the urine to give the aldehyde reaction. The correspondence has failed in either direction in some instances, that is, the intestine

[1] *Medicinische Woche*, 1901, No. 15.

[2] *Sitzungsber. d. Gesellsch. f. Morph. u. Physiol.*, 1903, 2, p. 32.

[3] *Zentralbl. f. inn. Med.*, 1905, No. 34, p. 833.

has sometimes contained considerable skatol while the urine has given no aldehyde reaction, and the reverse of these conditions has also been encountered. In about one-half of the urines from the anæmia group the Ehrlich aldehyde reaction has been obtainable, though not without the aid of heat. The strongest reactions I have observed have been obtained not in the case of the patients forming this group but in persons without marked anæmia, who were suffering from chronic disturbances of intestinal digestion. In the cases of anæmia now under consideration a marked aldehyde reaction of the urine has been less often obtained than a marked reaction for indican. It has also been evident that this aldehyde reaction bore no relation either to the indican reaction or to the phenol content of the urine.

The Dimethylamidobenzaldehyde Reaction of the Feces.

If one extracts human feces with an aqueous sodium chloride solution (2 grams of feces to 20 grams of 0.85 per cent. sodium chloride solution) the extract will usually yield a color reaction with a suitably prepared acid solution of Erhlich's aldehyde.[1] In the case of healthy children or adolescents on a mixed diet the color obtained is usually a light rose, and may be very faint. Between this light tint and a very deep cherry-red, all transitions are met.[2] It is noteworthy that the feces from the anæmia patients under consideration have regularly yielded very strong or intense Ehrlich aldehyde reactions, the only exceptions to this rule having been met with during periods of diarrhœa. It sometimes happens that a patient with chronic intestinal indigestion or a person on a strict proteid diet gives an intense Ehrlich aldehyde reaction. Normal adults on mixed diet give a moderate reaction and (in cases where the ethereal sulphates, phenol, and indican of the urine run low) often only a faint one.

The explanation of the chemical basis of the Ehrlich aldehyde reaction of the feces is not yet wholly satisfactory. Baumstark[3] thought it could be ascribed to the indol of the feces and based

[1] Water, 270 c.c., concentrated H_2SO_4, 30 c.c., Ehrlich's aldehyde, 15 grams.

[2] We have employed a graded color scale in order to record our results with some degree of accuracy.

[3] *Münch. med Wochenschr.*, No. 17, 1903; also, *Arch. f. Verdauungskrankh.*, ix, 1903.

a quantitative method for indol on the reaction. Bauer,[1] however, showed that the feces contain another substance which reacts with the aldehyde and claims that this substance is urobilinogen. I reached a similar conclusion independently, after noticing that the feces in one instance gave an intense red reaction after the indol had been distilled off. In other cases a strong reaction was obtained in spite of the fact that the feces were free from indol from the outset. That the reaction from this non-volatile part of the feces depends wholly on urobilinogen does not appear to me to have been convincingly shown. It is true, however, that one may reduce urobilin (Schuchardt's) with alkali and zinc dust and thus obtain a substance which gives a stronger and more characteristic Erhlich aldehyde reaction than the urobilin itself. Probably both urobilinogen and a skatol derivative are implicated in the Ehrlich aldehyde reaction of the urine, but it is possible that other substances are also concerned.

Acids and Bases of the Feces.

Some attention has been paid to the quantity of volatile acids present in the feces of anæmic and other patients and also to the volatile bases present. The titration values have usually been such as to show that the ammonia (of which the bases mainly consist) almost exactly neutralizes the acetic, propionic, and butyric acids of which the acid in the distillate mainly consists. The values for volatile acids and bases in anæmias have not shown uniform deviations from normal values. Nevertheless it is common to find rather high values for the volatile fatty acids of the feces of persons with "primary" pernicious anæmia. Such high values are not confined to cases of this sort but are frequently observed in the intestinal contents of persons suffering from chronic intestinal indigestion, associated with an excessive excretion of phenyl-potassium sulphate and indoxyl-potassium sulphate by the urine. In such instances of chronic intestinal indigestion with excessive putrefaction there is almost invariably present some degree of anæmia. The hæmoglobin may not be greatly reduced. It is not exceptional to meet with persons who show the intestinal conditions just mentioned, but whose hæmoglobin is not below 70 or 80 per cent. A careful examina-

[1] *Loc. cit.*

tion in such instances will, I think, often reveal the fact that there is more real anæmia than is indicated by a consideration of the hæmoglobin alone, there being in such cases unequivocal signs of a diminished volume of blood. In both these milder anæmias and in the more severe forms, it is characteristic to find the various signs (to be described later) of the presence of an excessive degree of what may be designated the saccharo-butyric type of putrefaction—a process attended by the excessive formation of butyric acid (perhaps also propionic, valerianic, and caproic). I have been able to show that in many of these cases of excessive intestinal putrefaction attending milder or more severe types of anæmia, the higher volatile fatty acids are considerably increased in amount.

The following table includes some of the results obtained from a study of fatty acids of the feces.

TABLE SHOWING THE QUANTITY OF VOLATILE FATTY ACIDS IN 100 GRAMS OF DRIED FECES (IN TERMS OF OXALIC ACID).

Clinical Data	Volatile Fatty Acids—Grams	Other Data
I. Observation on normal child on mixed diet, showing minimal indications of putrefactive intestinal decomposition.	0.095	
II. Observation on patient aged 16 with continuous fever (influenza) of 39°C–40°C and temporary increase in intestinal putrefaction. Mixed diet.	0.426	
III. Observation on adult patient with primary pernicious anæmia (Case XIII of tables).	0.3304	
IV. Observation on adult patient with primary pernicious anæmia (Case XII of tables).	0.44	Molecular weight of volatile acids = 71. Molecular weight of propionic acid = 72.
V. Observation on adult with primary pernicious anæmia. Very excessive intestinal putrefaction (Case III of tables).	0.23	Molecular weight of volatile fatty acids = 89.
VI. Observation on adult patient with excessive intestinal putrefaction (saccharo-butyric) and slight anæmia. Mixed diet, somewhat restricted.	0.57	Molecular weight of volatile fatty acids = 71.
VII. Observation on child aged 5, with chronic intestinal putrefaction (large belly type). Putrefactive products very excessive. Saccharo-butyric type. Moderate anæmia.	0.60	Molecular weight of volatile fatty acids = 85. Molecular weight of butyric acid = 84.

In this table are seen extreme values for the volatile fatty acids of the feces. In Observation I we are dealing with a normal child; in Observation VII, with a child with an extreme condition of intestinal putrefaction and there is good reason to think that the acid values for adults fluctuate rather widely and that they sometimes reach rather high figures temporarily in persons whose health is not greatly impaired. The three observations relating to primary pernicious anæmia do not reveal extremely high values for the acids, but they are in excess of what is generally found in healthy persons on similar, somewhat restricted diets. Perhaps one reason why the differences in the acid contents of the feces in health and disease are not more pronounced is because of the free absorption of these very soluble products, which are made to some extent even in normal digestion. It seems probable that the differences in acid production in health and disease are considerably greater than the above recorded observations would indicate. It should be noted that in Observation IV, the molecular weight of the acids corresponds closely to that of propionic acid, while that of Observation VII corresponds to butyric acid and that of Observation VI falls between the molecular weights for butyric and valerianic acids. Some experiments were made to determine whether the acid production of the mixed fecal bacteria is greater when these bacteria are obtained from persons with excessive intestinal putrefaction than when they are derived from normal persons. The results were not uniform. In some instances the volatile fatty acids were greater in amount in the case of the flasks inoculated from the putrefactive cases than in the case of any inoculated from normal persons. The sediment in these cases contained large numbers of strictly anaerobic putrefactive bacteria such as B. putrificus or B. aerogenes capsulatus and both organisms were sometimes present. Peptone bouillon was employed as a medium and B. aerogenes capsulatus grew freely in this only under special conditions. The failure to find a large production of volatile fatty acids does not therefore mean that anaerobic putrefactive bacteria have been absent from the feces but may mean merely that they were unable to grow under the given conditions. B. aerogenes capsulatus usually grows well in blood bouillon (rabbits' blood may be used) and

it would have been better if this medium had been employed.

A feature of interest in this connection is that the molecular weights of the fatty acids made by the bacteria from putrefactive cases tend to be higher than the molecular weights of the fatty acids derived from the action of flora derived from relatively non-putrefactive digestive tracts. Thus the volatile fatty acids from the flora of a case of excessive intestinal putrefaction gave a molecular weight of 73; the same weight was given by the acids obtained from the flora of a case of pernicious anæmia. Another case of excessive putrefaction gave bacteria which made acids with a molecular weight of 74. These results were all obtained on sugar-free media. On the other hand the flora from a child showing extremely low putrefactive processes generated acids having a molecular weight of 64.7 in peptone medium, and 61. 5 in peptone lactose (molecular weight of acetic acid being 60).

These and similar observations accord with the contention of Rodella[1] that anaerobic putrefactive bacteria tend to make the higher fatty acids. It was found also that a culture of B. putrificus (Bienstock) on peptone gave acids with a molecular weight of 86 . 8. A culture of B. aerogenes capsulatus (B. Welchii) gave acids which, expressed in terms of propionic acid, calculating from the weight of the barium salts, amounted to 0.257 gram per 100 c.c. of culture; expressed as a mixture of acetic and butyric acids, the yield was equivalent to 0.104 gram acetic and 0.153 gram butyric acid.

Sulphur Compounds.

Observations on the fresh feces from persons with "primary" pernicious anæmia have failed to show any peculiarities in respect to the presence either of mercaptan or hydrogen sulphide. Hydrogen sulphide could usually be detected but was not usually present in considerable amount. Repeated and careful tests (isatin-sulphuric-acid method) for the presence of mercaptans in no instance led to a positive result. This is of special interest in view of the fact to be emphasized later, that it is common

[1] "Sur la différentiation du 'Bacillus putrificus' (Bienstock) et des bacilles anaérobic tryptobutyriques (Achalme)," *Ann. de l'Inst. Pasteur*, xix, p. 804, 1905.

for the fecal bacteria from persons with pernicious anæmia to make mercaptan when grown on sugar-free peptone bouillon.

The Hydrobilirubin Reaction of the Feces.

A not unimportant indication of unusual conditions of bacterial activity in the intestine is an excessive hydrobilirubin reaction of the feces. This reaction, first described by Schmidt in 1895, is developed when one acts on certain kinds of feces in the fresh state by means of a concentrated watery solution of mercuric chloride. The marked and characteristic red color with yellowish fluorescence which appears under these conditions is believed to depend on a combination between the mercuric salt and hydrobilirubin (perhaps identical with urobilin). An analogous combination exists in Jaffe's zinc-chloride-urobilin compound. Both substances give the same spectroscopic picture—namely a band between the lines *b* and *E*.

In the course of a systematic examination of the feces from normal individuals and from many different pathological sources, it was noticed that the mercuric chloride reaction was strongest in persons suffering from intestinal disorders, especially in those with excessive intestinal putrefaction. The weakest reactions were found in the case of acholic stools and in the case of children and young adults presenting only slight indications of intestinal putrefactive decomposition (low ethereal sulphates, absence of indican and low phenol). The results in the anæmia cases are briefly summarized in the following table:

TABLE RELATIVE TO THE HYDROBILIRUBIN REACTION OF THE FECES

Case	Date of Examination	Degree of Reaction with $HgCl_2$	Behavior of Material on Exposure to Air
1	Mar. 30, '06 Apr. 1, '06	Very strong Strong Strong	
II	Mar. 21, '06 Mar. 28, '06	Very strong Very strong	Greenish; shows red-brown layer on exposure *Light brown; red layer on exposure*
III	Mar. 20, '06 Apr. 30, '06	Very strong Very strong Almost immediately	

Case	Date of Examination	Degree of Reaction with $Hg\ Cl_2$	Behavior of Material on Exposure to Air
IV	Dec. 18, '05	Strong	
	Jan. 5, '06	Strong	
VI	Mar. 17, '06	Strong	
	Mar. 26, '06	Moderate	Greenish; reddish on exposure
VII	Jan. 18, '06	Moderate	
	Apr. 21, '06	Faint	
VIII	Dec. 30, '05	Moderate	
	Jan. 9, '06	Moderate	
	Apr. 14, '06	Very strong	
X	Nov. 28, '05	Very strong	
	Dec. 2, '05	Very strong	
	Dec. 9, '05	Very strong	
	Dec. 13. '05	Strong	
	Dec. 18, '05	Strong	
	Feb. 1, '06	Moderate	
	Mar. 14, '06	Negative	Greenish with yellow streaks
XI	Dec. 19, '05	Strong	Yellow, changing to red on exposure
XII	Dec. 27, '05	Moderate	Turns reddish yellow on surface
	Jan. 2, '06	Strong	Light yellow; turns reddish on surface
	Jan. 12, '06	Moderate	
	Feb. 1, '06	Moderate	Turns reddish-brown
	Feb. 14, '06	Strong	Turns reddish-brown
	Mar. 17 '06	Strong	
	Apr. 5, '06	Very strong	
	Apr. 27, '06	Very strong	
XIII	Apr. 17, '06	Very faint Doubtful	
XIV	Jan. 4, '06	Moderate	Dull yellow; darker on surface
	Jan. 10, '06	Moderate	

Case	Date of Examination	Degree of Reaction with $HgCl_2$	Behaviour of Material on Exposure to Air
XV	Mar. 30, '06	Strong	Very light yellow, darkening on exposure
	Apr. 30, '06	Fairly strong	
XVII	May 5, '06	Intense immediately	

The table shows that in our cases of advanced anæmia it was usual to meet with a strong mercuric chloride reaction of the feces. This result is so frequent among cases of this kind that it becomes noteworthy. Very strong reactions were observed in some cases of moderate anæmia in which putrefactive decomposition in the intestine was extreme. The reaction doubtless depends on the reduction of bilirubin in the course of putrefactive decomposition in the intestine and may therefore be expected to run parallel to the reducing activity of the intestinal bacteria. A careful comparison of the reducing activity of the intestinal bacteria, as measured by the effect of the mixed fecal bacteria on various media colored with methylene blue, neutral red, and methyl violet, failed to demonstrate any close relationship of this kind, although in general the strongest mercuric chloride reactions were found in those cases in which the intestinal reduction was most active. There were, however, cases in which the bichloride reaction was feeble despite the fact that the intestinal bacteria were shown to be capable of reducing strongly. It seems likely that another factor is necessary to make possible a strong mercuric-chloride reaction, namely the presence of a sufficient supply of biliary coloring matter in the large intestine. For it is true that when the bile is cut off from the intestine, as in obstructive jaundice, the bichloride reaction may wholly fail and is usually feeble. The persistence of the reaction in slight degree, in some cases where autopsy has demonstrated the presence of complete obstruction of the biliary duct, has been referred by some writers to a slight secretion of biliary coloring matter through the intestinal walls, and this is perhaps the true explanation. If the presence of biliary coloring matter is essential to the production of the bichloride reaction, one would expect this reaction to be particularly intense when blood destruction is from any cause excessive and permits the escape

of exceptionally large quantities of bilirubin into the intestine. The mere introduction of an excess of bile pigment into the intestine does not, however, suffice to intensify the reaction, as I have satisfied myself by means of experiments on dogs. Apparently two factors are concerned in determining the formation of hydrobilirubin—the presence of a sufficient amount of bilirubin and the existence of conditions of bacterial activity permitting the rapid reduction of this coloring matter. Outside the body the reduction of bilirubin to hydrobilirubin by bacteria was a slow process in the experiments which I have thus far made.

In some cases the feces which gave a strong hydrobilirubin reaction showed a spontaneous and marked alteration in color on exposure to the air.

The relation of the hydrobilirubin reaction to the urobilinogen reaction of the feces with Ehrlich's aldehyde is a point of interest on which one cannot at present express an opinion. As already mentioned there are instances in which the feces, after distillation[1] of all the indol present (as shown by the aldehyde reaction, which is very delicate), still give a reaction *in the cold* with a dimethylamidobenzaldehyde solution. This reaction is believed to depend on the presence of urobilinogen. It has been fairly well marked in some of our anæmia cases but there is as yet no evidence that the reaction bears any definite relation to the hydrobilirubin reaction although the two substances urobilinogen and urobilin (hydrobilirubin?) are closely related chemically.

The Ethereal Sulphates.

It is generally admitted by physiological chemists that the ethereal sulphates of the urine are perhaps the best single index to the extent of putrefaction in the intestine, or, more accurately stated, to the degree of absorption of putrefactive products into the circulation. Physiologists differ, however, as to what constitutes an excessive excretion of ethereal sulphates, some laying stress on the absolute quantities excreted daily, others attaching especial importance to the ratio between ethereal and preformed sulphates. From a long experience in dealing with this question

[1]This distillation should be conducted in an atmosphere of carbon dioxide in order to prevent oxidation of the reacting substance which is sensitive both to air and to the action of sunlight.

I have learned to emphasize especially the necessity of considering the ratio of ethereal and preformed sulphates. It has been urged against this ratio that as the preformed sulphates are derived from proteid metabolism whereas the ethereal sulphates represent putrefactive decomposition, we are comparing incommensurate things when we use the ratio as a measure of putrefaction. It should, however, not be overlooked that in health, and still more in disease, the quantity of the putrefactive products bears a relation to the quantity of proteid ingested—an excess of proteids in the dietary being the most certain means of increasing intestinal putrefaction. Against the use of the absolute ethereal sulphate value as an index of putrefactive decomposition is the fact that in health this value fluctuates widely. If the exact conditions of diet and absorption were known, this absolute value would be useful but it is ordinarily quite impracticable to obtain the necessary data. On the other hand, I have been able to satisfy myself that when the ratio of ethereal and preformed sulphates falls below 10 one almost always meets with excessive quantities of phenol or indol in the feces. It will be seen from the table that the ratio of ethereal and preformed sulphates tends to run low in the anæmia cases studied—the tendency being very pronounced in Cases II, III, IV, VIII, IX, X, XII, and XV. These results are simply corroborative of the general proposition that the putrefactive processes were excessive in the intestinal tracts of the patients under consideration.

Hunter in his well-known work on pernicious anæmia has recorded results obtained from the study of the excretion of the ethereal sulphates in a case of pernicious anæmia.[1] The results obtained in this case are similar to those reported here. The absolute quantities of ethereal sulphate are not strikingly high, but on most occasions the ratio of preformed to combined sulphuric acid is high. Hunter concludes from his figures that the absolute amount of putrefaction occurring within the intestinal canal was not excessive, but that in proportion to the quantity of proteid food ingested putrefaction was distinctly excessive. He believes further that the relative putrefactive excess was not sufficiently great to be credited with being the

[1] *Pernicious Anæmia: Its Pathology, Septic Origin, Symptoms, Diagnosis, and Treatment.* London, 1901.

TABLE SHOWING EXCRETION OF ETHEREAL SULPHATES.

Case	Date	Preformed Sulphuric Acid. Grams	Combined Sulphuric Acid. Grams	Ratio
I	Mar. 23, '06	0.6675	0.2715	9.8
	Apr. 2, '06	2.0731	0.1289	16.0
II	Mar. 21, '06	0.7421	0.1720	4.2
	Mar. 25, '06	0.2952	0.0872	3.4
III	Mar. 21, '06	0.3091	0.0580	5.3
	Apr. 27, '06	1.1124	0.2816	3.9
IV	Dec. 18, 05	0.3005	0.1444	2.1
	Jan. 5, '06	0.5891	0.1080	5.4
VI	Mar. 20, '06	0.4615	0.1522	3.0
	Mar. 24, '06	0.5846	0.0462	11.6
VII	Mar. 15, '06	0.7947	0.1167	6.9
VIII	Jan. 9, '06	0.1265	0.0210	6.0
IX	Dec. 4, '05	0.1753	0.0353	5.0
X	Nov. 27, '05	0.9930	0.2220	4.4
	Nov. 30, '05	1.5075	0.1665	9.0
	Dec. 4, '05	1.4510	0.1050	13.9
	Dec. 7, '05	0.6831	0.0809	8.4
	Dec. 11, '05	1.368	0.1317	10.4
	Jan. 31, '06	0.6234	0.1164	5.4
	Mar. 14, '06	2.7538	0.3042	9.0
	Mar. 27, '06	1.5738	0.1972	8.0
XII	Dec. 26, '05	1.1677	0.1093	10.7
	Jan. 15, '06	0.8966	0.1680	5.3
	Jan. 31, '06	0.7063	0.1066	6.6
	Feb. 16, '06	0.7565	0.1115	6.8
	Mar. 3, '06	1.1764	0.1186	9.9
	Mar. 16, '06	1.236	0.0749	16.5
	Apr. 5, '06	1.4218	0.1092	13.0
	Apr. 27, '06	1.0859	0.0691	15.7
XV	Mar. 30, '06	1.2254	0.3046	4.0
	Apr. 4, '06	1.5867	0.3313	4.7

cause of the special symptoms of so well-marked a disease as pernicious anæmia. This latter conclusion is one to which I should not wish to commit myself on the grounds brought forward by Hunter, for while, as already stated, the ethereal sulphates are the best single index of the degree of intestinal putrefaction they give no clue to the character of the putrefactive products, on which the pathological significance of the putrefaction may in an important measure depend.

On the Production of Methyl Mercaptan and of Gas by the Mixed Fecal Bacteria from Advanced Cases of Anæmia.

As the character of physiological activities of the bacteria of the feces is obviously a matter of interest in any study of the flora of the digestive tract, and as these activities can hardly be pictured with success by any method involving the formation of a composite picture through the fusion of our conceptions of the physiological activities of individual bacterial species, an attempt has been made to learn something through the cultivation of the mixed bacterial flora on artificial media. These studies relate to the production of gas, ammonia and other bases, acids, indol, phenol, mercaptan, and hydrogen sulphide. It is desired to call attention here to only two manifestations of bacterial activity—the ability to make methyl mercaptan and the capacity to produce gas. These subjects have already been touched on in a preliminary way in this Journal[1] and the methods employed have been described with sufficient fulness.

The chief drawback to the use of this method in the study of intestinal disorders is that we have no guarantee that the mixed fecal flora introduced by inoculation into an artificial culture medium will develop there in the same way as in the intestine. Indeed it is certain that in many instances the dominant types of bacteria in the culture medium are not the same as in the feces. Still the method has a certain value for the reason that the decompositions observed bear a definite relation to the flora present in the digestive tract, although not one expressive of the exact conditions of putrefaction within the body.

[1]This Journal, i, p. 415 and p. 421, 1906.

The Production of Methyl Mercaptan by Fecal Bacteria from Persons with Advanced Anæmia.

What has been learned from a study of the mercaptan reaction in relation to the anæmia cases may be briefly summarized. In seven of the cases (Nos. I, IV, IX, X, XII, XV, and XVII) strong or intense reactions for mercaptan were obtained by means of the isatin sulphuric acid test. Some of these reactions are the strongest we have observed in the course of considerable experience with the method. In Case II there was apparently no tendency to mercaptan production and in some of the other cases the inclination of the fecal bacteria to make mercaptan when grown on peptone bouillon was apparently not considerable, although in many instances the observations were not sufficiently numerous to enable one to form a positive judgment.

As pointed out in a previous paper, the persistent tendency of fecal bacteria from adults to make mercaptan on a simple peptone medium is probably to be regarded as a pathological rather than a normal manifestation, although it is true that one sometimes finds apparently normal adults whose bacteria make enough mercaptan under the described conditions to give a fair isatin reaction. Especially in the case of bottle-fed babies have I frequently found instances in which the intestinal flora were capable of making mercaptan. A slight mercaptan production may give place to the production of larger quantities with the onset of a febrile disease. The fecal flora may produce mercaptan but very little hydrogen sulphide; in general, however, the bacteria which make an abundance of hydrogen sulphide tend to make methyl mercaptan.

The explanation of the mercaptan production which has been noted in disease and sometimes in health is not yet clear. B. putrificus (Bienstock) is the only micro-organism we have yet found which is capable (in pure culture) of making mercaptan from a peptone medium. There is as yet no evidence that the observed mercaptan production by fecal bacteria is the result of the action of putrificus, but the possibility has not been definitely excluded.

I am not disposed to attach much physiological or pathological significance to the formation of mercaptan by fecal bacteria.

This is partly because experiments on dogs with high enemata of methyl and ethyl mercaptan solutions repeated daily over long periods (25 to 50 c.c. of a one per cent. solution) have

TABLE SHOWING INTENSITY OF MERCAPTAN REACTION IN PEPTONE MEDIUM INOCULATED WITH FECAL BACTERIA FROM PERSONS WITH ADVANCED ANÆMIA.

No. of Case	Date	Mercaptan Reaction	Remarks
I	Feb. 5, '06	Moderate	Reaction completed in 10 min.
	Mar. 23, '06	Strong	" " "
	Mar. 24, '06	Intense	" " "
	Mar. 27, '06	Intense	" " "
	Mar. 31, '06	Strong	" " "
	Apr. 3, '06	Intense	" " "
	Apr. 6, '06	Strong	" " "
II	Mar. 22, '06	Negative	No development in 40 min.
	Mar. 27, '06	Faint trace	Reaction occurred in 15 min.
III	Mar. 21, '06	Faint trace	Reaction required 30 min.
	May 1, '06	Negative	No development in 20 min.
IV	Dec. 18, '05	Strong	Reaction required 5 min.
	Jan. 3, '06	Strong	Deep green after 10 min.
V	Jan. 26, '06	Moderate	
VI	Mar. 17, '06	Negative	Time allowed was 30 min.
	Mar. 26, '06	Strong	Time required was 7 min.
VII	Jan. 18, '06	Negative	Time allowed was 31 min.
	Mar. 15, '06	Trace	
	Apr. 22, '06	Strong	Time, 15 min.
VIII	Dec. 30, '05	Negative	3 days' growth
	Jan. 9, '06	Trace	
	Apr. 15, '06	Intense	Reaction occurred in 10 min.
IX	Dec. 4, '05	Strong	Olive green in 5 min.

No. of Case	Date	Mercaptan Reaction	Remarks
X	Nov. 30, '05	Strong	Time allowed was 5 min.
	Dec. 8, '05	Slight	
	Dec. 12, '05	Strong	
	Dec. 18, '05	Slight	
	Feb. 1, '06	Strong	Time allowed, 5 min.; culture two days old.
	Feb. 5, '06	Strong	
	Mar. 14, '06	Strong	Time was 10 min.
XII	Dec. 25, '05	Trace-strong	Trace appeared in 5 min., became strong in 30 min.
	Jan. 2, '06	Strong	Culture 1 day's growth
	Jan. 2, '06	Negative	2 days' growth in cystin medium
	Jan. 17, '06	Negative	
	Feb. 1, '06	Strong	Time, 5 min. on culture 4 days old
	Feb. 1, '06	Negative	
	Mar. 4, '06	Trace	Time allowed, 30 min.
	Mar. 18, '06	Intense	Time allowed, 7 min.
	Apr. 6, '06	Strong	Time allowed, 30 min.
	May 1, '06	Intense	Time, 10 min.
XIII	May 1, '06	Moderate	Time allowed, 50 min.
XIV	Jan. 3, '06	Negative	
	Jan. 10, '06	Negative	
	Feb. 5, '06	Intense	Reaction developed in 5 min. in a culture 2 days old.
XV	Mar. 30, '06	Strong,	Time allowed, 10 min.
	Apr. 6, '06	Strong	Time allowed, 25 min.
	May 1, '06	Negative	Time allowed, 20 min.
XVI	Dec. 1, '05	Trace	Time allowed, 20 min.
	Dec. 8, '05	Fairly strong	
	Dec. 15, '05		
	Dec. 22, '05	Strong	Time allowed, 10 min.
XVII	May 5, '06	Intense	Time allowed, 15 min.
	May 7, '06	Intense	Time allowed, 10 min.

failed to induce definite toxic manifestations. Moreover it appears to me by no means certain that methyl mercaptan is actually formed in the intestine (and absorbed therefrom)

in appreciable quantity by the bacterial flora which, outside the body, have been found able to make this sulphur compound. Although hydrogen sulphide is readily detectable in the freshly passed feces of many individuals with advanced anæmia, I have never been able to detect more than a trace of methyl mercaptan even in quite fresh material. This fact, though not conclusive, makes one question whether mercaptan is formed in any part of the intestinal tract in quantities that possess any pathological significance.

But notwithstanding the absence of evidence that mercaptans are factors in the production of intoxications, the ability of certain flora to make these substances outside the body, on a peptone medium, is of some biological interest and it has seemed desirable to state here the persistence with which mercaptan formation occurs under the action of bacteria derived from the intestines of certain anæmic patients. [1]

On the Restricted Formation of Gas by the Fecal Flora Grown on Sugar Bouillon.

Early in the course of the present investigation it was noticed that the fecal flora from certain individuals almost regularly fall far below the normal standard of activity in gas production on sugar-bouillon media. It was observed also that material derived from persons with pernicious anæmia and allied blood diseases is so apt to show the peculiarity of low gas production that it must be regarded as a feature of such affections, although by no means limited to them.

As already explained elsewhere[2], the mixed fecal flora from healthy adults on a mixed diet make considerable gas when grown on sugar bouillon for twenty-four hours, and the volume of gas made under fixed conditions is apt not to vary widely in the case of the same individual, if the dietetic conditions remain similar. In the observations made in my laboratory, the practice has been followed of inoculating four sugar-bouillon fermentation

[1] Dr. Rettger writes me that he has obtained a strong mercaptan reaction from the products of growth of the mixed fecal bacteria from three patients with pernicious anæmia. I have also observed another case of pernicious anæmia (not included in the above report) in which a very strong reaction was noted.

[2] This Journal, i, p. 415, 1906.

tubes (dextrose-, levulose-, lactose-, and saccharose-bouillon) with the material to be tested. Usually the gas production has been greatest in the lactose tube and least in the saccharose tube. The column found in each tube was measured and the total gas production in the series of four tubes was recorded. It was found that in normal individuals the average gas production for the four tubes is about 100 millimeters.[1] Among healthy individuals on a mixed diet, the fecal flora seldom yield less than 65 millimeters of gas in the four tubes. The bacteria derived from persons with digestive disorders, on the other hand, very frequently give not more than 60–75 millimeters of gas in the four tubes. In the case of persons suffering from blood diseases, a very much greater restriction in gas production has been noted, as will be seen by reference to the table. The tendency to low gas production is especially notable in Cases I, II, VI, VII, VIII, X, XIV, and XVII. In Case XII, in which eight observations were made, the gas production fell below 73 millimeters only on three occasions (45, 60, and 66 millimeters).[2] In Case XV, also, we have three observations which fall within the normal limits (79, 103, and 136 millimeters), this case being one of secondary anæmia associated with pronounced putrefactive conditions. In looking over the table of recorded results

[1] The anaerobic limb of our fermentation tubes measures about 95 millimeters.

[2] It should be stated that after the manuscript of this paper had been sent to press the gas production by the fecal flora from Case XII fell to 25 millimeters (in the four sugar-bouillon tubes). This fall in gas production was coincident with a period of relapse in which the red blood cells and hæmoglobin declined considerably (although only temporarily). At the same time the microscopical fecal fields showed a very marked increase in bacteria of the type of B. aerogenes capsulatus and a falling off in organisms of the B. coli type. Typical B. aerogenes capsulatus was found abundantly on the highly anaerobic agar plates prepared at this time. The presence of this organism in unusual numbers was furthermore proved by the gas production noted in an incubated rabbit which had been injected with an exceptionally dilute suspension of the fecal bacteria (1–50). Moreover it was impossible to recover bacteria of the B. coli group from gelatin plates made at this period. Finally, it should be noted that the fecal bacteria grown in peptone bouillon produced methyl mercaptan in unusual abundance, judging by the intensity of the isatin reaction.

it will be seen that there are other instances in which gas production has fallen within the normal limits.

A feature of much importance to the interpretation of the recorded results is the presence or absence of diarrhœa. Repeated observations have shown that the occurrence of diarrhœa tends to increase the gas-forming activities of the fecal flora. The explanation of this fact is, I think, obvious. It has been found that the gas production, in the four sugar-bouillon tubes which has been incited by the action of pure cultures of B. coli derived from human sources resembles closely in volume and chemical character the gas production by the mixed fecal bacteria from normal adults. This fact, viewed in connection with the observed growth of organisms of the B. coli type in the fermentation tubes, has led to the inference that a large part of the gas production on the part of the fecal flora is due to the activities of organisms of the B. coli type. It is known that these bacteria tend to die as they approach the rectum from higher levels of the intestine, and that living gas-producing bacteria (B. coli and lactis aerogenes types) are more numerous in the upper than in the lower colon. It can easily be shown that conditions which induce a rapid passage of the intestinal contents through the gut bring down increased numbers of living gas-forming, Gram-negative bacteria. The relative increase in B. lactis aerogenes is probably a factor in the greater gas formation.

A comparison of the pictures presented by the Gram-stained, microscopical, fecal fields with gas production induced by the bacteria which constitute these fields, leads to one definite conclusion. It is that Gram-positive fields, containing relatively few or poorly preserved micro-organisms of the B. coli type are associated with restricted gas production almost without exception. On the other hand, fecal material showing Gram-negative fields, made up of well-preserved bacteria corresponding morphologically to the type of B. coli, rarely fail to produce gas abundantly.

The observed failure to make gas in normal amount in pernicious anæmia, I refer to a diminution in the number of living micro-organisms of the B. coli type in the feces, though there is no proof that in some instances this factor may not be reinforced by the inhibitory activity of other types of micro-organisms.

TABLE SHOWING INTENSITY OF GAS PRODUCTION IN PEPTONE MEDIUM INOCULATED WITH FECAL BACTERIA FROM PERSONS WITH ADVANCED ANÆMIA.

No. of Case	Date	Amount of Gas Production. Millimeters	Remarks
I	Feb. 5, '06	97	Diarrhœal movement
	Mar. 20, '06	(1) 26	
		(2) 10	
	Mar. 24, '06	5	
	Mar. 27, '06		
	Mar. 31, '06	54	
	Apr. 3, '06		
	Apr. 6, '06	62	
II	Mar. 22, '06	62	
	Mar. 27, '06	35	Coli type poorly preserved
III	Mar. 20, '06	62	Coli type fairly numerous; fairly preserved
	Apr. 29, '06	114	
IV	Dec. 18, '05	155	Diarrhœa
	Jan. 5, '06	45	Coli type present; preservation poor
V	Jan. 26, '06		Coli type abundant; poorly preserved
VI	Mar. 17, '06	54	Coli type only moderately abundant
	Mar. 26, '06	56	Coli type only moderately abundant
VII	Jan. 18, '06		Coli type poorly preserved
	Mar. 15 '06		
	Apr. 20, '06	43	Blood condition much improved
III	Dec. 30, '05	95	Diarrhœa
	Jan. 9, '06	70	
	Apr. 14, '06	63	

No. of Case	Date	Amount of Gas Production. Millimeters	Remarks
IX	Dec. 4, '05	160	Diarrhœal movement
X	Nov. 28, '05	25	
	Nov. 30, '05	30	
	Dec. 5, '05	100	Diarrhœal movement
	Dec. 8, '05		
	Dec. 12, '05		
	Dec. 18, '05		
	Dec. 28, '05	60	
	Feb. 1, '06	30	
	Feb. 5, '06		
	Mar. 14, '06	20	
XII	Dec. 25, '05	85	Semi-solid movement
	Jan. 2, '06	85	Movement soft
	Jan. 17, '06	112	Soft movement from high enema
	Feb. 1, '06	45	Movement formed
	Feb. 14, '06	76	Movement formed
	Mar. 4, '06	60	Partly formed movement
	Mar. 18, '06	66	Partly formed movement
	Apr. 6, '06	102	Soft, unformed movement
	Apr. 28, '06	86	Semi-solid movement
XIII	Apr. 18, '06	116	Fluid movement
XIV	Jan. 3, '06	35	
	Jan. 10, '06	38	
	Feb. 5, '06	53	
XV	Mar. 30, '06	103	Soft, unformed movement
	Apr. 4, '06	79	Partly formed movement
	Apr. 30, '06	136	
XVI	Dec. 1, '05	20	
	Dec. 8, '05	100	
	Dec. 15, '05	150	
	Dec. 22, '05	95	
XVII	Apr. 30, '06	15	Semi-solid, pasty movement
	May 4, '06	25	

The considerable or normal or even excessive gas production noted in some cases of pernicious anæmia I think may be reasonably ascribed to the presence of gas-producing organisms (of the B. coli type) incidental to diarrhœa. This is evidently true of many of the cases which have come under my observation. It is probable, however, that other bacterial influences are sometimes at work. In a few fermentation tubes in which there has been high gas production and in which organisms of the B. coli type have been scanty, there has been evidence of multiplication of organisms morphologically like B. aerogenes capsulatus (B. Welchii). This organism is a rapid and abundant gas-maker on sugar-media and may have been responsible for the gas formation in the cases mentioned. If this be the case, it shows that a normal volume of gas in the fermentation tubes does not necessarily mean that the fermentation has been carried on by the normal gas-makers of the intestine—*i.e.*, by organisms of the B. coli and B. lactis aerogenes group. Ordinarily B. aerogenes capsulatus does not grow well on sugar bouillon in the absence of blood or fresh animal tissues (such as liver) and it is not yet clear to what conditions the occasional observed growth has been due.

It is believed that the phenomenon of restricted gas production by the fecal bacteria is one of much biological and pathological interest and that the method here employed in the study of gas production will prove of clinical value. The phenomenon of restricted gas production is by no means limited to the bacteria derived from persons with severe anæmias but is observed in lesser degree in many digestive disorders.

I have several times observed it in the course of fever and think the explanation here is probably the same as in the cases of digestive derangement—*i.e.*, the partial elimination of living colon bacilli from the feces—a condition of colon scarcity.

In a recent paper which promised to prove significant for the understanding of bacterial conditions in the intestinal tract, Conradi and Kurpjuweit advanced experimental evidence to show that the obligate colon bacilli of the human intestine produce substances capable of holding in check the development of other species of bacteria,—for example the typhoid and paratyphoid organisms. To these inhibitory substances they gave

the name "autotoxines," as it appeared that the colon bacilli were themselves subject to the inhibitory influence exerted by the substances produced by them. If such inhibitory powers could have been shown to reside in the colon bacilli, it would have thrown an important light on our knowledge of the antagonisms between intestinal bacteria, and especially on the role of the colon bacillus group in helping to exclude saprophytic forms. The work of Conradi and Kurpjuweit has, however, been recently subjected to criticism and it seems clear that the phenomena of inbibition which they described as being dependent on autotoxines are open to other explanations.[1] It appears from these criticisms that the phenomena of inhibition attributed to the formation of autotoxines are probably due, at least in part, to the exhaustion of the nutrient media in which the colon bacilli have grown. Moreover the inhibition of the growth of typhoid bacilli and other bacteria appears to have been much less complete than was supposed by Conradi and Kurpjuweit. It is a matter of considerable importance in connection with the present study to know whether a free growth of the obligate colon bacilli in the intestine operates in such a way as to check the development of Bacillus aerogenes capsulatus. It is certainly true that a greatly diminished representation or a complete elimination of typical colon bacilli from the feces is a feature of many cases of severe anæmia and it is possible that this partial or complete disappearance of colon bacilli constitutes a condition especially favorable for the infection of the digestive tract by Bacillus aerogenes capsulatus.

The Examination of Sediments from Fermentation Tubes.

In speaking of the study of the Gram-stained fecal bacterial fields, some emphasis is laid on the value of this method in gaining an acquaintance with the dominant bacterial flora of the lower part of the intestine. There is perhaps no better intro-

[1] See especially the criticisms of Moro and Murath, "Ueber die Bakteriellen Hemmungsstoffe des Säuglingsstuhles," *Wien. klin. Wochenschr.* xix, p. 371, 1906; also F. Passini, "Die bakteriellen Hemmungsstoffe Conradis und ihr Einfluss auf das Wachstum der Anaerobier des Darmes," *ibid*, p. 627; and R. Oebius, "Ueber spontane Wachstumshemmung der Bakterien auf künstlichen Nährboden," *Med. Klinik*, ii, p. 598, 1906.

duction to the bacterial conditions that prevail in the intestine than the careful examination of a series of microscopical fields from representative parts of the material derived from the lower bowel. The method has, however, an important limitation which should be clearly recognized. It is that the Gram stain gives us no positive indication as to which bacteria are living and which are dead. It is important to be informed on this subject, for if the apparently dominant organisms in a field are really for the most part dead and autolysing, it is obvious that they do not constitute the physiologically dominant variety. Especially in the case of micro-organisms of the B. coli group is it desirable to have some information on this point, since these organisms probably exert a protective action against certain invaders of the intestinal tract. It is often possible to gain some knowledge as to the living and dead varieties of fecal bacteria by examining the sediments from the fermentation-tube cultures which have been employed in studying the gas production, indol production, etc., of the intestinal flora. The results of such study have developed the following facts: (1) organisms of the B. coli group may fail to grow freely in the anaerobic limb of the fermentation tube on glucose bouillon, or may not grow at all, after inoculation with fecal flora which appear by the Gram stain to contain many (though perhaps poorly preserved) organisms of the B. coli type. The inference in such cases is that living B. coli were in reality not well represented in the feces, although in some cases another possibility presents itself—namely that the bacteria of this type though not dead have been inhibited by other species. Among our patients with advanced anæmia the fermentation tube has in several instances pointed to the absence of B. coli in the fecal contents,—always in cases that have shown restricted gas production. Among normal or nearly normal persons who have been used for control observations, a failure of these organisms of the B. coli type to make gas has not been observed. In a case of diabetes, however, with Gram-positive fields, B. coli failed to grow—again with small gas production. The absence of living B. coli in the feces of some patients with pernicious anæmia is further indicated by the failure of representatives of this group to appear on litmus-gelatin plates.

(2) The positive diplococci which are normally seen in the

feces grow readily in the sugar-bouillon fermentation tubes and are apt to be well represented in the sediment both from normal and pathological cases.

(3) Streptococci are sometimes found in great abundance in the fermentation-tube sediment. This is especially apt to happen in the case of material which microscopically shows the presence of a considerable number of streptococci; but it may also happen that there is an abundant and dominant growth of streptococci in cases where the microscopical fields have failed to call attention to its presence.[1]

(4) An abundant growth of an organism having the morphological characters of B. aerogenes capsulatus was a frequent occurrence in the tubes inoculated from patients with advanced anæmias. This was observed in Cases I, II, III, VI, VII, VIII, IX, and XVI, and also in a case of diabetes, material from which had shown capsulatus-like organisms in the Gram-stained fields. On the other hand, capsulati were not ordinarily observed to grow in the tubes inoculated from the feces of normal persons selected as controls. The growth of B. aerogenes capsulatus was generally most abundant in the lactose-bouillon tubes.

Action of the Mixed Fecal Bacteria on Milk.

Among the first observations made in the course of this research was the fact that sterilized milk undergoes a peculiar "stormy fermentation" when inoculated with the mixed fecal flora from certain individuals, whereas this is not induced by bacteria from other sources. In this fermentation the casein is coagulated, broken up into small fragments, and undergoes some degree of peptonization, while there is at the same time a rapid

[1]In one instance in which the apparently normal feces contained very many leucocytes and only a few coccal forms in the fields, the fermentation-tube sediments showed the growth of streptococci only, colon organisms failing to grow in most of these tubes. It afterwards developed that the patient had a small dental abscess, containing streptococci, the pus from which had for two years been passing into the stomach. The chief clinical conditions were anæmia, mental depression, and loss of weight. I am indebted to Prof. James for the opportunity of studying these conditions. A very similar observation was made in the case of a young woman who developed mucous colitis during convalescence from measles.

and voluminous liberation of gas. The meaning of the phenomenon was not at first clear, but it now seems certain that it is referable to the action of bacteria of the B. aerogenes capsulatus type. This view is borne out by the following facts: (1) B. aerogenes in pure culture sets up the "stormy fermentation" just mentioned; (2) the fecal flora setting up this type of fermentation in milk were derived almost exclusively from persons in whose stools micro-organisms of the B. Welchii type were found in considerable abundance (including nearly all the cases of "primary" pernicious anæmia in which this clinical diagnosis appeared justified); (3) the fecal flora from persons showing slight signs of intestinal putrefaction and few or no capsulati in the feces failed to give the characteristic active fermentation of milk. These results, however, which differentiate between the action of bacterial suspensions containing few capsulati and those containing many, are obtainable only by inoculating the fermentation tubes with small quantities of the suspension. If one inoculates large quantities of the suspensions the number of capsulati contained in the normal material will sometimes be sufficient to induce the typical change in milk in the course of twenty-four hours.

BACTERIOLOGICAL OBSERVATIONS

(In Conjunction with Herbert C. Ward.)

The necessity of obtaining definite knowledge of the bacterial inhabitants of the digestive tract, in conditions attended by evidence of excessive putrefaction within this tract, is self-evident. In the case of our group of anæmias this necessity was further emphasized by the frequent recurrence, within the group, of a phenomenon, calling for a bacteriological explanation—the phenomenon, already described, of small gas production in sugar bouillon. The problems relating to the bacterial flora of the intestine are so beset with technical difficulties and difficulties of interpretation that even their proximate solution (in the sense in which any life-phenomena are soluble by scientific methods) is a task of the not very near future. What we have to offer on the subject of the bacteriology of the intestine in anæmia is put forward with a realization of its inadequacy, but in

the belief that facts thus far collected represent a necessary step in progress.

There are several methods by which we have sought to obtain knowledge of the bacterial conditions in the intestinal passages of persons with apparently primary, profound anæmia, in the hope of finding in these conditions something to account for the evidences of putrefactive decomposition and of restricted fermentation, which we have found to characterize these cases. There are three general methods of approach from which, up to the present time, we have had the most encouragement. These are: (1) the microscopical study of the Gram-stained fecal fields; (2) the microscopical study of the fermentation-tube sediments derived by inoculating certain media with the mixed fecal bacteria; and (3) the study of the bacteria cultivated under strict anaerobic conditions from fecal suspensions subjected to pasteurization. These methods and their results will be separately described and their relation to each other will be considered.

Study of the Gram-stained Fecal Fields.

The microscopical examination of the Gram-stained fecal fields (counterstained with saffranin or carbol-fuchsin) has proved in several respects a serviceable method. It enables the observer to roughly classify the bacteria of a given field as strongly positive, positive, mixed, negative or strongly negative. This is a real gain, since it gives at once a clue to the relative importance of organisms of the colon bacillus type—a feature which, as will later be made evident, is of prime importance. The recognition of the dominant bacterial types is much facilitated by this study, for it usually enables the practised observer to distinguish with a serviceable degree of accuracy organisms of the following types: B. coli, B. pyocyaneus, B. aerogenes capsulatus, B. bifidus, various diplococci and staphylococci, yeast organisms, etc. The presence of spore-bearing organisms and of free spores can also be made out. The presence of capsules can also be made apparent in some instances.

In the following tables are recorded some features of typical fecal fields from a group of anæmia patients and from a group of "normal" persons, chosen almost at random.

The features which find expression in the tables are the char-

acter of the fields with respect to the Gram stain, the abundance and state of preservation of organisms of the B. coli type, the presence of positive coccal forms, the presence of spore-bearing, capsulatus-like bacilli,[1] and the presence of oval spores, such as are under some conditions formed by B. Welchii. Other bacterial characters of the fields have been for the time being ignored. The above characteristics of the fields were selected for the following reasons. It had been noted that in certain cases the fecal flora are unable to ferment sugar bouillon with full gas production. As gas production in fermentation tubes inoculated with normal adult feces appears to be mainly due to organisms of B. coli and B. lactis aerogenes type (B. Welchii not growing readily even in the anaerobic limb of the tube, on sugar bouillon without blood or animal tissues) the character of the fields with regard to B. coli became a question of interest. Negative fields with an abundance of well-preserved organisms of B. coli type one would expect to see in those feces that give an abundance of gas; positive fields with relatively few coli forms in a poor state of preservation one would expect to find associated with deficient gas production on sugar bouillon. Both these relations have been found to hold true in general. Attention was called to the importance of positive cocci by observation on the sediments of fermentation tubes in which both cocci and B. coli had been grown. The cocci clearly tend to retard the gas-producing action of B. coli on sugar bouillon. This is a result in harmony with the similar observations lately made by Heinemann[2] in another connection. The reason for paying special attention to the frequency of organisms of the B. Welchii type is that on beginning the study of the strict anaerobes from an anæmia patient conjointly studied with Professor Theobald Smith, it was found that there occurred regularly on the blood-agar plates large

[1] I have come to believe that these Gram-positive, spore-holding bacilli, which are morphologically indistinguishable from B. aerogenes capsulatus do not as a rule belong to this class but to a group of aerobic and physiologically very different class of organisms. The reasons for this opinion will appear later.

[2] "The Significance of Streptococci in Milk," *Journ. of Infect. Dis.*, iii, p. 173, 1906. It is here pointed out that B. aerogenes var. lacticus is held in check and ultimately stopped by the presence and ascendency of streptococcus lacticus.

numbers of this organism and extremely few other strict anaerobes of the spore-forming class.[1] It was shown by Hirshberg[2] in Professor Welch's laboratory that B. aerogenes capsulatus is a very frequent inhabitant of the intestinal tract of man and other animals[3] and it had been shown by Welch[4], Howard,[5] and others, that local lesions of the gastro-enteric tract occur which are attributable to this organism. It therefore appeared desirable to inquire more closely than had been done into the occurrence of B. Welchii in the intestinal contents in health and disease. There soon appeared indications that there are numerous instances in which B. Welchii is very abundant (as compared with its occurrence in normal individuals) in the feces. The study of the question from the standpoint of its occurrence in persons with advanced apparently primary anæmias assumed increasing interest and the results on the fecal fields thus far noted are embodied in the table.

If we compare the table of normals with that of the anæmias the following differences may be noted: (1) the tendency of the normal fields is toward Gram-negativeness while that of the anæmia fields is toward Gram-positiveness; (2) the representation and preservation of B. coli are poor in most of the anæmia fields as compared with the representation of this group in the normal fields[6]; (3) positive coccal forms are distinctly more abundant in the anæmia group than in the normals (in one of the cases [VII, March 15] positive diplococci were extremely abundant

[1] Our attention was originally and previously directed to B. putrificus (Bienstock) as a cause of excessive intestinal putrefaction, but this line was abandoned because it appeared unpromising.

[2] "Distribution of Bacillus Aerogenes Capsulatus (Bacillus Welchii, Migula)," *Jaurn. Bost. Soc. for Med. Sci.*, v, p. 369, 1900–1901.

[3] We have found that the feces of tigers may consist almost wholly of free spores at least a portion of which develop into the vegetative form of B. aerogenes capsulatus.

[4] *Bull. Johns Hopkins Hosp.*, Sept., 1900.

[5] *Contributions to the Science of Medicine Dedicated by his Pupils to William Henry Welch*, Baltimore, 1900, p. 461.

[6] It is often difficult to form a positive judgment from the microscopical appearances as to the condition of B. coli in the fields, but with practice it becomes possible to predict in many instances the behavior on culture media.

and made up the dominant bacterial type); (4) bacilli of the type of B. Welchii are distinctly more abundant in the anæmia fields than in the normal ones, the difference in this respect between the two sets of cases being pronounced; (5) spore-bearing organisms of the capsulatus type were rarely seen in the normal fields and were not a feature in the anæmia cases, although in three instances (Cases I, V, and X) they were present; (6) free oval spores, suggesting capsulatus spores, were not usually abundant either in the normal cases or in the anæmias, but in three of the latter cases (Cases I, V, and X, the cases showing spore-holding bacilli) they were numerous and often were seen in clumps and chains.

It is especially important to note that there are four instances (Cases VII, X, XV, and XVI) in which the fields showed an abundance of B aerogenes capsulatus when the patients first came under observation and that at a later period, after treatment was begun, there was in these same cases a noteworthy falling off in their number. At the same time there was noted in three of these instances an opposite change, *i.e.*, an increase, in regard to negative bacteria of the B. coli type. The observations included in the table which illustrate the alterations in the bacterial flora are in harmony with observations on other material from the same cases which have not been included in the table.

It is desirable to state wnat is meant when speaking of the 'capsulatus type" in the fecal fields. The term is used to refer to rather large, plump, Gram-positive bacilli, usually with slightly rounded free ends and square-cut opposed ends, sometimes showing capsules—organisms morphologically typical of B. Welchii. They frequently occur in pairs, end to end. More frequently they occur singly. Occasionally they appear as long threads. The term is here further used to include bacilli somewhat smaller and thinner than the typical B. Welchii, but having in other respects the same morphological characters and the same staining properties. These latter organisms, which are sometimes numerous in the fields, are included in the "capsulatus type" because it is known that under certain conditions the true Bacillus aerogenes capsulatus assumes these characters and because our more detailed studies of colonies

TABLE RELATING TO OCCURRENCE OF B. COLI TYPE, B. AEROGENES CAPSULATUS TYPE, AND POSITIVE DIPLOCOCCI IN CASES OF NORMAL INDIVIDUALS OR OF PERSONS WITH AILMENTS IN WHICH ANÆMIA WAS SLIGHT OR ABSENT

CASE.	DATE	GRAM-POSITIVE OR NEGATIVE.	B. COLI TYPE.	COCCI.	CAPSULATUS TYPE	SPORE-HOLDING FORMS.	FREE SPORES.	REMARKS.
Normal child, age 8 years.	April 14, 1906.	Mixed.	Very numerous; well defined.	Fair number, chiefly negative.	Found small numbers.	Very few.	Few.	
Normal girl, age 15 years.	Dec. 27, 1905.	Negative tendency.	Abundant; many fairly preserved.	Negative cocci rather abundant.	Very few.	Not seen	Few.	Detritus abundant. A few negative double ended spore-holders.
Normal man, age 65 years.	Jan. 8, 1906.	Negative strong	Abundant; a few well preserved.	Negative cocci fairly numerous	Extremely few.	Not seen.	Small numbers.	
Normal man, about 21 years.	March 3, 1906.	Negative.	Poorly preserved; abundant	Considerable numbers, negative.	Extremely few.	Not seen.	Some few.	Much detritus.
Normal man, about 30 years.	April 2, 1906.	Strongly negative.	Very numerous; well preserved.	Negative forms in considerable numbers.	Very few.	Not seen.	Fairly abundant.	Very abundant detritus
Normal man, age 24 years. Temporary indigestion.	April 7, 1906.	Mixed.	Fairly abundant; poorly preserved.	Moderate numbers; mostly positive	Moderately abundant.	Not seen	Small numbers.	Much detritus. Patient was showing marked putrefactive changes in urine.
Man aged 55 years, chronic gastritis	Dec. 12, 1905.	Negative.	Abundant; many well preserved.	Moderate numbers, negative.	Few.	Not seen	Moderate numbers.	
Normal man, aged 26 years	Jan. 16, 1906.	Negative.	Abundant, but fair preservation.	Few, positive and negative.	Very few.	Not seen.	Moderate numbers.	
Boy aged 17 yrs. Severe diabetes.	Feb. 7, 1906. Feb 2, 1906.	Mixed. Essentially the same.	Abundant; not well preserved as the preceding.	Very few.	Moderately abundant. " "	None.	Very few. Numerous.	
Woman aged 50 years. Diabetes.	April 12, 1906.	Negative tendencies.	Very abundant; fairly preserved.	Few.	Few.	Not seen.	Few.	
Robust man aged 42 years.	March 20, 1906.	Positive tendency.	Abundant; very well preserved.	Very few	Presence doubtful; small positive bacilli very abundant.	None.	Not seen	Case of universal eczema. Evidences of intestinal putrefaction extremely slight.

of this sort have shown them to be abundant gas producers and otherwise to conform to the characters of B. aerogenes capsulatus. There is, however, another Gram-positive organism, smaller than that just mentioned, and uncapsulated, which is less strictly anaerobic than B. Welchii and makes little or no gas, which must be carefully distinguished from the "capsulatus type." It has not yet been fully studied by us, but probably corresponds to the plain forms of Tissier's B. bifidus. Furthermore it seems wise at present to speak of the capsulatus type, in referring to the fecal fields because there is reason to believe that there are sub-varieties of Welch's gas bacillus, sub-varieties based mainly on differences respecting the difficulty of sporulation, upon pathogenic qualities, hæmolytic properties, indol production, rapidity of gas production in animals, etc.

Finally it must be stated that there is sometimes found in the feces an organism morphologically so similar to the true B. aerogenes capsulatus as to be indistinguishable from it.[1] It is evident

[1] The pseudo aerogenes organism which is here in question possessed the following characters in a case where it had been isolated from the feces of a person suffering from excessive intestinal putrefaction. The organism did not differ in any appreciable way in size or morphology from typical B. aerogenes capsulatus. It was Gram-positive and proved to be a facultative anaerobe capable of abundant growth aerobically in peptone bouillon. On ordinary fluid media, it formed a pellicle on the surface which after a time fell to the bottom. It was found to sporulate readily after a few days' growth on agar. It was sluggishly motile. The organism produced no gas on dextrose, levulose, lactose, or saccharose bouillon. It grew luxuriantly on agar slants and plates forming a dense, opaque, white, dry, and wrinkled surface. It produced acid on dextrose and saccharose. On peptone bouillon it produced neither hydrogen sulphide nor mercaptan nor indol nor skatol. It produced volatile fatty acids, apparently chiefly butyric acid, after several days' growth on peptone bouillon. It also produced ammonia. Two cubic centimeters of the bouillon culture ineculated into the femoral vein of a rabbit produced no gas and failed to give rise to the very characteristic decomposition induced by B. aerogenes capsulatus. In this experiment the organism in a state of involution was found in moderate numbers in the spleen but it had failed to grow in the liver. Two cubic centimeters of the bouillon culture injected intraperitoneally into a guinea-pig gave rise to no symptoms. The organisms had no hæmolytic action upon blood nor did they show any capacity to reduce hæmoglobin. The spores formed by this aerobic organism were oval and were located midway between the middle and end of the bacillus. No capsule was observed when the organism was grown on ordinary media.

from this fact that we cannot safely rely upon the appearance of the Gram-stained fecal fields as a certain index of the presence of B. aerogenes, although it appears probable that in most cases in which the feces show an abundance of bacilli suggesting this organism the true B. aerogenes capsulatus is in reality present in numbers pointing to an unphysiological condition of the bacterial flora.

The Growth of Micro-organisms of Capsulatus Type on Sugar-Blood-Agar Plates under Anaerobic Conditions.

Perhaps the most satisfactory method of determining the presence of organisms of the capsulatus type in the feces is that which consists in growing these organisms on sugar-blood-agar plates. This method not merely gives evidence as to the presence of the bacteria in question, but can be utilized to gain a rough idea of the numbers present in the feces. In the present instance no effort was made to get an idea of the actual numbers of capsulatus present in the feces, but the suspensions of fecal material used for inoculating the sugar-agar plates were so employed as to give a fair basis for comparison of the numerical results in pathological and normal conditions.[1] The results here given relate to the spores and spore-bearing varieties of bacteria in the feces and not to vegetative forms, for the suspensions containing the intestinal flora were subjected for twenty minutes to a temperature of 80° C. in order to eliminate vegetative forms of bacterial life. In each case duplicate series of plates were made, one series being incubated under aerobic conditions, the other under anaerobic conditions. A satisfactory state of anaerobiosis within the plates was obtained with the aid of a hydrogen stream from gas stored in the compressed form in tanks. The pyrogallic acid method of securing absorption of oxygen was employed as an auxiliary procedure to get rid of the last trace of oxygen and to indicate the conditions within the bell-jar.

The results obtained in this way are given in the accompanying

[1] The fecal suspensions were made in approximately the same way in the normal and pathological cases, and the inoculation of the agar plates was made with quantities of the fecal suspension designed to avoid an excessive number of anaerobic colonies on the plates.

tables, where the presence of aerobic, spore-bearing organism is indicated in addition to the presence of anaerobes. This double procedure is of value because it has occasionally revealed the presence of an organism resembling capsulatus in morphology, and which makes oval spores, but grows aerobically as well as anaerobically and does not make gas in the manner characteristic of the true B. aerogenes capsulatus.[1]

Inspection of the tables shows clearly the difference in results yielded by material from normal persons as compared with that derived from our advanced anæmias. The number of organisms of the capsulatus type found on the anaerobic plates made from material from persons with normal digestion—that is with little indication of putrefactive decomposition—is small where such organisms were found at all. One of the persons selected as a control showed a considerable number of capsulatus colonies (about 100 anaerobic colonies being found on the plate) but it should be noted that in this individual putrefactive disturbances are of frequent occurrence and are readily induced by slight errors in diet. A considerable number of capsulatus-like organisms were observed on the plates made from an adolescent patient with advanced diabetes and signs of impending coma. In a number of instances the plates from the controls showed no colonies suggestive of capsulatus. Plates made from the anæmia cases, on the other hand, almost without exception showed the presence of capsulatus-like colonies and often these were numerous. In one of the cases observed by us a distinct fall in the number of capsulatus-like organisms on the plates was observed as the patient convalesced and the same observation was quite independently made on this patient by Professor Theobald Smith. Unfortunately the conditions did not permit us to study all our cases in this manner for in many instances they came under notice only after treatment had been commenced and the original blood picture had been altered.

In conclusion it may be said that the plate method has confirmed the observations already made from the study of the Gram-stained fecal bacterial fields, that organisms of the capsulatus type occurred in much greater abundance in the feces of persons with advanced anæmia than in those of normal persons.

[1] The characters of this organism are given on page 39, footnote.

The designation "capsulatus type" was applied to colonies on the basis of the appearance of the colonies and the morphological character of the bacteria composing them. In some cases these bacteria were examined with respect to motility, gas production in the fermentation tube, gas production in the dead rabbit, the production of spores, etc.

ANÆMIA CASES.

Case No.	Aerobic Plates		Anaerobic Plates	
	No. Colonies Present	Capsulatus Type	No. Colonies Present	Relative No. of Capsulatus Type
I	50	?	60	50-60 per cent.
II	Many	None		Majority examined
III	Few	"	75	50-60 per cent.
IV	30	Few?	400	Cap. type abundant
VI	Few	?	40	50-60 per cent.
VII	"	None	100	50-75 " "
VIII	"	"	125	15-20 " "
IX			Many	Majority examined
X	20	50 per cent.	"	" "
XII	Very few	None	40	60-80 per cent.
XIV	Few	"	20	20-30 " "
XV	?	"	15	20-25 " "
XVI	Very few	"	100	40-50 " "

CONTROL CASES

Cases	Aerobic Plates		Anaerobic Plates	
	No. of Colonies Present	Capsulatus Type	No. of Colonies Present	Relative No. of Capsulatus Type
Chronic intestinal indigestion; male aet. 6	Few	None	?	Few
Normal child, age 2 mos.	"	"	Many	None
Normal child, age 6 mos.	"	?	Few	"
Chronic indigestion, malnutrition and slight anæmia; girl, 8 yrs.	Few	?	Few	None
Anæmic baby, about 10 mos.	Many	Few	25	Less than 5 per cent.
Normal child, age 13 yrs.	Many	None	Many	Very few
Man, age 30 yrs.	25	Few	100	20–30 per cent.
Man, age 42 yrs.	20	"	10	None
Girl, age 10 yrs.	10	None	Few	"
Man, age 25 yrs.	40	"	40	"
Advanced diabetes; boy, age 16 yrs.	40	"	75	40–50 per cent.
Splenic leucæmia, male 22 yrs.	Very few	?	20	None

The Incubation Test for B. Aerogenes Capsulatus in the Feces.

In their classical study of B. aerogenes capsulatus, Welch and

Nuttall[1] made the striking observation that the gas bacillus has the faculty of producing gas abundantly in the blood, organs, and tissues of rabbits killed a few minutes after intravenous injection. Here the blood and tissues of the rabbit act as a peculiarly favorable culture medium for the growth of the gas bacillus, the bacteria having been thoroughly spread by the blood through the body and the conditions being anaerobic. Welch and Nuttall made use of their procedure to isolate the gas bacillus and to demonstrate its ability to make gas on a proteid medium containing little sugar.

It seems singular that so little use has been made by subsequent investigators of this ingenious and extremely valuable method of studying the gas bacillus. Acting on the suggestions carried by the paper of Welch and Nuttall, we have used the method not merely as an aid in the identification of B. aerogenes capsulatus but also (with somewhat unexpected success) as a means of determining whether the gas bacillus is present in considerable numbers in the feces.

The conditions found at autopsy after twenty-four hours' incubation of a rabbit previously injected intravenously with a pure culture of B. aerogenes capsulatus, have been so fully described by Professor Welch that it is unnecessary to add anything to his description aside from emphasizing the fact that the almost intolerable odor of putrefaction which is developed during the incubation experiment is dependent in part on the production of butyric or a closely allied acid.[2] It should also be added that an odor very similar to that characteristic of the incubation experiment can be observed in the feces of some persons with chronic disturbances of digestion, and frequently in persons with advanced anæmias associated with irregular diarrhœal conditions.

The incubation method of Welch and Nuttall has apparently never been employed in connection with the study of the human feces but I believe it has here an important clinical application. Although it is true that B. aerogenes capsulatus can be isolated from the feces of a majority of adult individuals, including very many who are in excellent health, it is also true that there are wide

[1] *Bull. of the Johns Hopkins Hosp.*, iii, p. 81, 1892.

[2] I do not know of an equally impressive example of the ability of micro-organisms to induce rapid putrefactive decomposition.

The designation "capsulatus type" was applied to colonies on the basis of the appearance of the colonies and the morphological character of the bacteria composing them. In some cases these bacteria were examined with respect to motility, gas production in the fermentation tube, gas production in the dead rabbit, the production of spores, etc.

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XII	Very few	None	40	60-80 per cent.
XIV	Few	"	20	20-30 " "
XV	?	"	15	20-25 " "
XVI	Very few	"	100	40-50 " "

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Anæmic baby, about 10 mos.	Many	Few	25	Less than 5 per cent.
Normal child, age 13 yrs.	Many	None	Many	Very few
Man, age 30 yrs.	25	Few	100	20–30 per cent.
Man, age 42 yrs.	20	"	10	None
Girl, age 10 yrs.	10	None	Few	"
Man, age 25 yrs.	40	"	40	"
Advanced diabetes; boy, age 16 yrs.	40	"	75	40–50 per cent.
Splenic leucæmia, male 22 yrs.	Very few	?	20	None

The Incubation Test for B. Aerogenes Capsulatus in the Feces.

In their classical study of B. aerogenes capsulatus, Welch and

Nuttall[1] made the striking observation that the gas bacillus has the faculty of producing gas abundantly in the blood, organs, and tissues of rabbits killed a few minutes after intravenous injection. Here the blood and tissues of the rabbit act as a peculiarly favorable culture medium for the growth of the gas bacillus, the bacteria having been thoroughly spread by the blood through the body and the conditions being anaerobic. Welch and Nuttall made use of their procedure to isolate the gas bacillus and to demonstrate its ability to make gas on a proteid medium containing little sugar.

It seems singular that so little use has been made by subsequent investigators of this ingenious and extremely valuable method of studying the gas bacillus. Acting on the suggestions carried by the paper of Welch and Nuttall, we have used the method not merely as an aid in the identification of B. aerogenes capsulatus but also (with somewhat unexpected success) as a means of determining whether the gas bacillus is present in considerable numbers in the feces.

The conditions found at autopsy after twenty-four hours' incubation of a rabbit previously injected intravenously with a pure culture of B. aerogenes capsulatus, have been so fully described by Professor Welch that it is unnecessary to add anything to his description aside from emphasizing the fact that the almost intolerable odor of putrefaction which is developed during the incubation experiment is dependent in part on the production of butyric or a closely allied acid.[2] It should also be added that an odor very similar to that characteristic of the incubation experiment can be observed in the feces of some persons with chronic disturbances of digestion, and frequently in persons with advanced anæmias associated with irregular diarrhœal conditions.

The incubation method of Welch and Nuttall has apparently never been employed in connection with the study of the human feces but I believe it has here an important clinical application. Although it is true that B. aerogenes capsulatus can be isolated from the feces of a majority of adult individuals, including very many who are in excellent health, it is also true that there are wide

[1] *Bull. of the Johns Hopkins Hosp.*, iii, p. 81, 1892.

[2] I do not know of an equally impressive example of the ability of micro-organisms to induce rapid putrefactive decomposition.

differences in the number of capsulati habitually present in the case of different individuals. There are some young persons between the ages of five and twenty years from whom it is either very difficult to obtain B. aerogenes capsulatus by plating or whose movements give no evidence whatever of its presence. If we prepare a suspension of the feces from such individuals by grinding 1 gram of the fresh material with 9 c. c. of 0.85 per cent. salt solution and filtering through absorbent cotton, we can inject intravenously one or two cubic centimeters of this suspension into a rabbit and then incubate the quickly killed rabbit for five hours at 37° C. without obtaining evidence of the abundant presence of the gas bacillus. On opening a rabbit which has been thus incubated, one finds none of the signs of the activity of the gas bacillus—no accumulation of gas in the liver or vessels or in the connective tissues or serous cavities. Moreover smears made from the liver blood or the auricular blood either do not show the presence of capsulatus at all or these organisms are present only in small numbers. If, however, the foregoing experiment be made with the fecal material derived from a patient with pernicious anæmia or from a person suffering from a capsulatus diarrhœa, one generally gets an entirely different result. At the end of five hours the liver is soft and friable, crepitates between the fingers, and on section shows the presence of many bubbles of gas. There may also be a small accumulation of gas in the peritoneal cavity. Smears from the hepatic blood and from the auricular blood swarm with organisms of the capsulatus type. The spleen also contains such organisms in great numbers.

The following protocols are instructive in this connection.

Experiment 1. One c.c. of a filtered fecal suspension, from a normal person 16 years of age, almost free from putrefactive products in the urine, was injected into a rabbit which was immediately killed by a blow on the neck. After five hours' incubation, the animal was examined. Abdomen distended slightly from distension of large intestine; liver firm, slightly friable and free from gas. A stained liver smear shows a few short bacilli (not capsulatus). Heart's blood shows short bacilli in abundance, rarely a bacillus of capsulatus type.

Experiment 2. Two c.c. of filtered fecal suspension from a healthy man (æt. 42) recently recovered from universal eczema, were injected intravenously into a rabbit which was then promptly killed by a blow on the neck. Animal incubated for 5 hours at 37°C. On examination, no

odor of butyric decomposition; liver firm and without gas bubbles. Smear from heart's blood showed a few bacteria of capsulatus type.

EXPERIMENT 3. Two c.c. of filtered fecal suspension from a healthy breast-fed baby were infused intravenously into a rabbit which was then promptly killed. Examination after 5 hours' incubation at 37° C. reveals no odor of butyric putrefaction and liver is firm and free from gas. Smears from the heart's blood show it to be free from bacteria of any kind.

These experiments may be contrasted with the following:

EXPERIMENT 4. Two c.c. of filtered fecal suspension from an anæmic baby with irregular diarrhœa (Case XVI of tables) were infused intravenously into a rabbit which was then promptly killed and incubated for 5 hours at 37° C. On examination the tissues gave a strong butyric acid odor. Liver soft, friable, and filled with bubbles of gas. Smears from heart's blood show a great abundance of bacteria of the capsulatus type.

EXPERIMENT 5. One c.c. of filtered fecal suspension from a patient with pernicious anæmia (Case VII of tables) was infused intravenously into a rabbit which was then incubated for 5 hours at 37° C. On examination the abdomen was slightly distended. Characteristic butyric odor. Liver crepitant, contains a few obvious gas bubbles. Bacilli of capsulatus type abundant in heart's blood, in almost pure culture. Capsulati also abundant in liver.

EXPERIMENT 6. Two c.c. of filtered fecal suspension from a patient with pernicious anæmia (Case I of tables) whose feces contained a great abundance of free (capsulatus?) spores were injected intravenously into a rabbit which was incubated at 37° C. for 24 hours. At autopsy the animal was greatly distended with gas, and bloody fluid was oozing freely from nose, mouth, etc. Gas escaping from abdominal cavity burns with blue flame. Extremely offensive odor of butyric decomposition. Tissues in advanced state of putrefactive liquefaction. Blood from heart shows bacilli of capsulatus type to be extremely abundant. Most of these were Gram-positive and occurred characteristically in diplobacillus form, but there were also many long threads which were doubtless capsulati. Gram-negative forms also occur and of these one variety was especially prominent. This was a long organism bearing a large spore near either end. It is probably to be regarded as a young form of capsulatus about to undergo division midway between the spores. This organism was Gram-negative.

The foregoing experiments are typical of a large group and show plainly enough the difference in capsulatus activity in the case of material derived from normal and pathological feces. It appears to be true that a 10 per cent. suspension of feces does not excite an active formation of gas in the liver, if the material

injected has been derived from persons free from intestinal derangements, and with scanty evidences of putrefactive products in the urine. On the other hand, material from persons whose stools contain an abundance of capsulatus (including a large proportion of persons suffering from "primary" pernicious anæmia) induces with great regularity the peculiar alterations in incubated rabbits which have been already described. The short period of time (four to six hours) which is required to bring about the liberation of gas in the liver is a point worthy of notice. Another fact of interest and importance is that this gas production is very characteristic of B. aerogenes capsulatus. Experiments made with pure cultures of B. putrificus, with the bacillus of malignant œdema, and with an aerobic spore-bearer resembling B. aerogenes capsulatus entirely failed to show the liberation of gas in the liver or the multiplication of these organisms in incubated rabbits.

The selective action of the dead organism for B. aerogenes capsulatus is certainly a striking feature. The infusion of any fecal suspension means the introduction of many varieties of living bacteria. Yet after a few hours of incubation in the dead rabbit the number of micro-organisms in the blood has been narrowed either to capsulatus alone or to capsulatus and one or two companions—often positive diplococci, sometimes spore-bearing bacilli resembling capsulatus but not positively identified. A longer period of incubation usually eliminates from the blood all organisms except those of the capsulatus type. The initial bactericidal power of the blood and cell juices may suffice to kill many of the bacteria of the feces, while the strict anaerobic conditions, so necessary to the multiplication of capsulatus, in itself cuts out many varieties.

It is believed that the more refined application of this method to the study of the feces will prove of clinical value in several directions. It may also prove of utility in the study of milk. If, as seems probable, B. aerogenes capsulatus is really identical with the granulo-bacillus-immobilis liquefaciens of Grassberger and Schattenfroh, (as is claimed by some observers and denied by others, including Fraenkel), this method may prove helpful in connection with the study of some diarrhœal diseases which have been attributed to the use of infected milk.

There is reason to think that the fecal suspensions from many persons in good health would induce gas production in incubated rabbits, since the feces of many apparently normal persons contain moderate numbers of B. aerogenes capsulatus. The difference, as regards the outcome of the incubation experiment, between the flora of these persons and the flora of persons with capsulatus infections is probably one of degree and not of kind. Some idea of the relative numbers of capsulati present might perhaps be gained by determining the smallest volume of a given fecal suspension that will just suffice to induce the distinctive gas production in incubated rabbits within a given time.[1]

Pathogenicity.—Opinions differ rather widely as to the pathogenic properties of B. aerogenes capsulatus for man and animals. Professor Welch,[2] who first discovered the organism in connection with human surgical infections, was disposed to regard it as not being ordinarily highly pathogenic. In reference to healthy rabbits he says we cannot regard this bacillus as being pathogenic under ordinary conditions. Dunham,[3] who, in the course of his clever investigations on B. aerogenes capsulatus, discovered its ability to make spores, ascribes a greater degree of pathogenic action to the micro-organism with which he worked—these bacteria also having been derived from cases of surgical infection.

[1] In one set of experiments, it was found that 8 c.c. of a fecal suspension (1 gram in 10 c.c. of 0.85 per cent. salt solution) could be intravenously infused without any development of gas on incubation of the infused rabbit. Larger volumes of the suspension could not be infused without causing death from mechanical causes. The material employed was obtained from a healthy bottle-fed infant in whose fecal fields no organisms even remotely resembling B. aerogenes capsulatus could be seen. Free spores were also apparently absent. On the other hand, the infusion of a fecal emulsion from a patient with pernicious anæmia gave the typical gas production in the liver when a weight equivalent to one-twentieth of the material employed in the foregoing experiment was used. Even a quantity of feces equivalent to one-fiftieth that used in the first series of experiments gave rise to some gas in the liver after incubation for twenty-four hours at room temperature.

[2] "A Gas-Producing Bacillus (Bacillus Aerogenes Capsulatus, Nov. Spec.) Capable of Rapid Development in the Blood-Vessels after Death," by Wm. H. Welch and Geo. A. F. Nuttall, *Bull. of the Johns Hopkins Hosp.*, No. 24, July–August, 1892.

[3] "Report of Five Cases of Infection by the Bacillus Aerogenes Capsulatus (Welch)," *Bull. of the Johns Hopkins Hosp.*, No. 73, April, 1897.

Grassberger and Schattenfroh, whose granulo-bacillus saccharobutyricus immobilis liquefaciens was derived from milk and is regarded by Welch, Kamen, and others as identical with B. aerogenes capsulatus, found it to be non-toxic for guinea-pigs. Kamen[1] although unable to obtain powerful toxins on ordinary culture media, regards B. aerogenes capsulatus as capable, by itself, of exciting inflammatory purulent processes. It is certain that there are various strains of B. aerogenes capsulatus as regards pathogenicity and that the different results obtained by different investigators with respect to that feature are due to this fact. Cultures of B. aerogenes capsulatus made by Mr. Ward from the feces of a young man with slight digestive derangement and from material derived from a case of pernicious anæmia were injected into the breast muscles of pigeons (according to a suggestion from Dr. Flexner, who had found these animals to be especially susceptible). The cultures set up a localized necrotic inflammation with gas production in the connective tissues. Death occurred, apparently from toxæmia, within twenty-four hours. The application of these facts to capsulatus infection of the intestine is not now clear. It is certain that the pathogenicity of B. aerogenes capsulatus is different for different strains isolated from the human digestive tract. Thus, Professor Theobald Smith sent us an organism (anaerobe xxxi) which he isolated from the stool of a patient with pernicious anæmia and which proved to be much less pathogenic for guinea-pigs than the typical form of B. aerogenes caspulatus. This organism differed but slightly from the typical bacillus in its morphology, but showed the important peculiarity of being non-hæmolytic. It fermented the various sugars, but the gas production was less abundant than in the case of the typical gas bacillus. The gas production and decomposition induced in an incubated rabbit were also less pronounced than in the case of the typical B. aerogenes capsulatus.

Agglutination.—A single observation was made by Mr. Ward on the blood serum from a patient with pernicious anæmia (and capsulatus infection of the intestine) with respect to a possible

[1] "Zur Aetiologie der Gasphlegmone," *Centralbl. f. Bakt. etc.*, I Abt., Orig., xxxv, No. 6, pp., 554, 686, 1904.

agglutination action. The results were entirely negative.[1] It is interesting to note in this connection that Kamen[2] obtained no agglutinative action from the serum of rabbits which had been immunized with the gas bacillus. Positive results have, however, been recently obtained by Werner[3] who employed a special technical procedure in the immunization of the rabbits which served as experimental animals. The immune serum caused agglutination of the homologous gas-phlegmon bacilli (derived from a gas liver found in a fatal case of wound infection) in a dilution of 1:1000. Passini[4] obtained positive results in some of his cases not only with homologous strains but also with unrelated ones. In this case, however, the agglutinative action of the immune serum was less marked than in the case of the sera obtained by Werner. It is evident that there is still much to be learned in relation to the immunizing action of B. aerogenes capsulatus and that such an action in man may yet be discovered.

Summary of the Leading Characteristics of Micro-organisms of the B. Aerogenes Capsulatus Type Derived from Human Feces.

The following is a summary of the leading characteristics of the micro-organisms which have been referred to in this paper as belonging to the B. aerogenes capsulatus "type." By no means all of these characteristics have been established for the suspected capsulatus micro-organisms from each case of saccharo-butyric putrefaction which has been studied, but a sufficient number of characters have been determined to show that we have been dealing with bacteria either identical with those described by Welch under the name, B. aerogenes capsulatus, or so closely affiliated as to fully justify us in classifying them as examples of this micro-organism. The expression "capsulatus type" is used because there are sub-varieties of the micro-organism described by Professor Welch. The most important sub-variety

[1] Prof. Theobald Smith tells me that he obtained negative results in agglutinative tests made with the blood of a patient with pernicious anæmia who showed large numbers of B. aerogenes capsulatus in the feces.

[2] *Loc. cit.*

[3] "Die Agglutination bei Gasphlegmonbacillen," *Arch. f. Hyg.*, liii, p. 128, 1905.

[4] *Münch. med. Wochenschr*, li, p. 1283, 1904.

at present known to us is the one above described as being non-hæmolytic in action and less pathogenic for guinea-pigs than the common form of the organism. The capacity of the bacillus to hæmolyse or *its failure to do* so may prove to be especially significant in relation to the etiology of anæmia.

The organisms we have classed as belonging to the B. aerogenes capsulatus type are large, plump, usually straight bacilli, which, as they occur in the feces, can usually be shown to be provided with a capsule. Organisms which have developed in a living or dead rabbit always acquire capsules. The organisms occur very often in pairs, end to end, sometimes singly; sometimes in chains; sometimes as threads, which may be nearly straight or sharply bent on themselves. The ends of adjacent bacilli are slightly rounded or squared, though not so sharply squared as in the case of anthrax bacilli. They are immobile when viewed in hanging drops. Spore formation occurs with difficulty; *i.e.*, chiefly under very special conditions, such as on a medium containing blood serum or within the body of an animal. Occasionally spore formation is seen in blood-agar colonies, the bacilli from which in other respects conform to the characters of B. aerogenes capsulatus. On sugar-bouillon, gas formation is abundant and rapid, twice as much gas (or more) being formed in twenty-four hours as is usually formed by organisms of the B. coli group. The gas consists of from one-third to one-half carbon dioxide, the remaining gas consisting mainly of hydrogen.[1]

[1] Prof. Theobald Smith gives the following gas formula for B. aerogenes capsulatus: $\frac{H}{CO_2} = \frac{2}{1} = \frac{3}{2}$

The following table illustrates the approximate ratio of hydrogen and carbon dioxide:

Source of Micro-organism	Medium	Height of Gas Column in Millimeters (in 24 hours)	$\frac{H}{CO_2}$
1. Young man with slight digestive derangement	Milk	87	$\frac{2}{1}$
" "	Dextrose-bouillon-blood.	7	$\frac{6}{1}$

The gas production in incubated rabbits is very rapid and is associated with a characteristic sweetish, sickening odor of butyric acid mixed with some unknown constituent or constituents. The gas obtained from the peritoneal cavity and connective tissues gives the hydrogen "bark" and burns with a blue flame. The liquefaction of muscles, liver, etc., is remarkably rapid in such

Source of Micro-organism	Medium	Height of Gas Column in Millimeters (in 24 hours)	$\frac{H}{CO_2}$
2. Same case as No. 1	Milk	75	$\frac{2}{1}$
" " "	Dextrose-bouillon-blood	80	$\frac{2}{1}$
3. Pernicious anæmia (Case *IX* of tables)	Milk	60	$\frac{3}{1}$
" "	Dextrose-bouillon-blood	75	$\frac{2}{1}$
4. Pernicious anæmia (Case XII of tables)	Milk	70	$\frac{5}{2}$
5. Pernicious anæmia (Case VI of tables)	Milk	100	$\frac{3}{1}$
" "	"	85	$\frac{5}{3}$
6. Pernicious anæmia (Case VIII of tables)	Milk	90	$\frac{2}{1}$
7. Same case	Milk	84	$\frac{5}{3}$
8. Aerobic capsulate bacillus	Milk	87	$\frac{5}{3}$
9. Milk bacillus	Milk	90	$\frac{2}{1}$
10. Milk bacillus	Milk	85	$\frac{5}{3}$
" "	"	85	$\frac{5}{3}$

incubated rabbits. Grown on pasteurized milk, the bacteria in question induce rapid gas formation ("stormy fermentation") with disruption of curds into small masses. They do not quickly produce hydrogen sulphide or methyl mercaptan on ordinary sugar-free media but may perhaps make these sulphur compounds more readily on milk. Grown in fermentation tubes containing blood bouillon, they rapidly liberate hæmoglobin.[1] The organisms are strictly anaerobic and many of their colonies on blood agar appear after two or three days as minute points which lie beneath the surface and develop into fuzzy spheres. These spherical colonies often have dark centres. The micro-organisms induce inflammatory necrotic changes with gas formation when injected into susceptible animals, such as pigeons.

As first shown by Prof. Theobald Smith, B. aerogenes capsulatus usually grows readily on bouillon in the closed arm of the fermentation tube provided small bits of sterile, fresh tissue are introduced into it. The liver of the guinea-pig may advantageously be used. The presence of the tissue probably favors the growth in two ways: by furnishing a constituent of the medium necessary for the growth of the organism, and by inducing a more strict condition of anaerobiosis by the reducing activity of the cells.

On the Significance of Excessive Saccharo-butyric or Capsulatus Fermentation and Putrefaction in the Human Intestine.

Evidence has been advanced in the preceding pages to show that the regular presence of B. aerogenes capsulatus (or organisms of this type) in large numbers in the intestinal tract is a characteristic of certain cases of advanced apparently primary anæmias, whereas in ideal conditions of human digestion it is present in small numbers only or is not detectable by ordinary methods. It has further been shown that in the group of anæmias in question the representation of B. coli in the feces has in most instances been unsatisfactory. We are now in a position to discuss the biological meaning of this overgrowth of organisms of the capsulatus type and subordination of the B. coli group.

[1] As already noted in the preceding pages, there is a non-hæmolytic variety of B. aerogenes capsulatus.

If we cultivate B. aerogenes capsulatus on sugar bouillon we find that it is a large producer of gas (mainly hydrogen and carbon dioxide) and that it makes butyric and closely related acids in abundance, while the formation of lactic acid is small. On media which contain very little sugar but much proteid, the organism is still able to make gas in considerable amounts, though less freely than on a sugar medium, in which the liberation of gas is remarkably rapid. In nearly sugar-free media the gas bacillus produces butyric acid and the quantity of this in old cultures may be surprisingly great. Ammonia is formed at the same time and serves to neutralize at least in part the acid which is simultaneously made. The organism may apparently also produce a large quantity of indol on a suitable medium. Thus in a sterilized egg-meat medium after a growth of one month at 37° C., 100 c.c. of the filtered culture contained 16 milligrams of indol, but no skatol.[1] The egg and meat had undergone gradual solution and peptonization. Professor Theobald Smith tells me he regards the absence of indol production as characteristic of most strains of B. Welchii, although other strains produce it (for example one derived from a rabbit gave indol).

More important for the pathologist than any of these substances is the formation of a moderately hæmolytic substance or substances by the gas bacillus. Evidence of such substances was obtained in a five-day culture of capsulatus in blood bouillon. One-half of one cubic centimeter of the filtrate from this culture induced hæmolysis in a suspension of rabbit's red cells prepared by Ehrlich's method, the filtrate having been carefully neutralized to the litmus point. The same result was obtained in the case of red cells from a large Rhesus monkey. Treatment of this filtrate in an exhaustion apparatus very slightly reduced the hæmolytic action; heating to 70° C. for one hour reduced it still further; but even boiling did not wholly destroy it.

In order to determine whether this hæmolytic action was dependent in part on volatile ammonium compounds, the capsulatus filtrate was rendered distinctly alkaline with sodium carbonate and concentrated under reduced pressure at a low temperature for the removal of ammonia. The filtrate was then

[1]This culture contained besides B. aerogenes capsulatus small numbers of a diplococcus.

restored to its original volume by the addition of 0.85 per cent. salt solution. It was found that the hæmolytic action of the fluid was somewhat diminished but not lost.

The hæmolysing action of B. aerogenes capsulatus is very clearly shown in fermentation tubes containing sugar-blood bouillon which have been inoculated with pure cultures. A free liberation of hæmoglobin occurs in twenty-four hours or less. A similar result is seen in the case of an aerobic organism closely resembling B. aerogenes capsulatus in morphology. The bacillus of malignant œdema does not exert a hæmolysing action under similar conditions. B. putrificus was found to reduce hæmoglobin, but this change is much less marked in the case of B. aerogenes capsulatus.

There are other indications that B. aerogenes capsulatus makes a hæmolytic substance or substances. Rabbits injected with pure cultures of B. aerogenes capsulatus and then incubated at 37° C. soon show indications of hæmolysis, whereas control animals subjected to the same procedure do not exhibit an equal degree of hæmolytic change in the same period of time. This corresponds with the observation that advanced hæmolysis is usually noted in persons who at autopsy show signs of general invasion of the gas bacillus.

We have then in the gas bacillus an organism which gives rise to a distinctive type of decomposition, characterized by abundant gas formation, the production of butyric acid, and the formation of hæmolytic substances of partly unknown nature. On many media the organism produces a slightly sweetish, sickening odor, which is highly characteristic and which may be intense.

In persons whose intestinal contents hold the living gas bacillus in large numbers we find definite evidence of the saccharobutyric type of fermentation, especially the production of gas in the colon (or higher up) and the formation of butyric acid. It has already been pointed out that butyric or allied volatile fatty acids are formed in abundance in some cases of pernicious anæmia in which flatulence is also common. This saccharobutyric type of fermentation may probably persist, associated with notable flatulence, for many years without the development of a marked anæmia. When dependent on B. aerogenes

capsulatus, the flatus may have the peculiar sickening, sweetish odor which I have just mentioned or a garlic-like odor noted in certain cultures of capsulatus.

It is not yet clear whether the organism makes a substance capable of exciting an acute inflammation of the ileum or colon or whether preceding mechanical or chemical irritation is necessary to enable the organism to multiply rapidly and excite further inflammation. Healthy monkeys may be fed considerable numbers of capsulati without developing signs of inflammation in the intestine, although such feeding is followed by an increase in these organisms in the feces. Two monkeys fed on gas livers from incubated rabbits infused with pure cultures of B. aerogenes capsulatus developed soft stools temporarily. Such experiments are, however, quite different from the experiment of introducing capsulati into a digestive tract already somewhat inflamed and irritable in consequence of preceding infections. The ability of B. aerogenes capsulatus to cause an inflammatory necrotic process in the muscles of guinea-pigs and pigeons, which was noted by Dr. Flexner many years ago, is of interest in this relation. It appears probable that B. aerogenes capsulatus is often the cause of slight inflammatory or perhaps even necrotic changes in the mucous membrane of the intestine. Howard[1] has described instances of superficial necrosis of the mucous membrane of the stomach and intestine, associated with the presence of capsulatus in abundance. These necrotic areas most often lie beneath the folds of the valvulæ conniventes and may occur with gas cysts. It does not seem likely that B. aerogenes capsulatus is responsible for severe acute inflammatory lesions of the intestine, but it is probable that its activities will account for the subacute enteritis that is so often present in cases that show large numbers of the bacilli in the stools. It is certain that there are many instances of acute diarrhœa associated with very free capsulatus multiplication and such diarrhœas are common in persons with severe primary anæmia. In one of our cases the disease set in soon after a period of intestinal diarrhœa contracted during the summer, and this

[1] *Contributions to the Science of Medicine, Dedicated by his Pupils to William Henry Welch on the 25th Anniversary of his Doctorate*, Baltimore, 1900, p. 461.

diarrhœa has been etiologically connected with the capsulatus infection.

The saccharo-butyric putrefactive process in the intestine, when active, is associated with an abundant liberation of gas, consisting largely of hydrogen. It therefore appears safe to attribute to B. aerogenes capsulatus much of the intestinal flatulence which is noted in persons suffering from digestive derangements characterized by the presence of capsulatus in great abundance. A breast-fed child (in the case of Dr. Holt), suffering from chronic capsulatus diarrhœa, was greatly troubled by flatulence until it received a milk containing less sugar. The ability of capsulatus to liberate hydrogen, even on media containing little carbohydrate material is one evidence of exceptional reducing power and I am disposed to connect this reducing power with an important though somewhat obscure characteristic of the feces in advancd anæmias.

It has already been noted that among our cases of anæmia there were few which did not give a strong mercuric chloride reaction in the feces, a reaction attributable to hydrobilirubin. As I have met with the strongest bichloride reactions in cases where capsulatus was very abundant in the feces, I am inclined to believe the reduction of bilirubin to hydrobilirubin is effected in these cases with the help of capsulatus. It has not yet been possible, however, to induce the reduction of bilirubin to hydrobilirubin experimentally by means of capsulatus activity, possibly because this reduction requires a symbiotic action not yet reproduced.[1]

The fecal extracts obtained from many persons with "primary" pernicious anæmia or with certain chronic intestinal derangements may exhibit distinct hæmolytic action toward the red cells of rabbits and monkeys, and in some cases this action is pronounced.[2] On the contrary the fecal extracts from healthy children on mixed diet may exhibit no appreciable hæmolytic action. The cause of the hæmolytic action just mentioned is

[1] Probably two factors are necessary to secure a highly excessive hydrobilirubin reaction: (a) excess of bilirubin in the bile, (b) excessive reducing action of bacteria in the intestine.

[2] In cases where the fecal extracts are deeply colored it is difficult to judge accurately of the hæmolytic action.

not yet clear. Indol may be present in considerable quantities in the intestine and its solutions exhibit a slight hæmolytic action for rabbits. Ammonium compounds are also frequently present in moderate amounts, and it is well known that such compounds have hæmolytic properties. In saccharo-butyric putrefaction ammonium butyrate may perhaps be formed in sufficient amount to cause some hæmolysis within the intestinal capillaries, and to contribute to the hæmolytic action of the fecal extracts. But it is also possible that, as already stated, B. aerogenes capsulatus forms some complex organic hæmolytic substance. Much more careful investigation of this point is necessary before drawing any conclusions as to its possible pathological significance.

The relation of capsulatus to the formation of indol in the intestine is not clear. Ordinarily the organism appears not to be an indol producer. In some of the anæmia cases, indol formation in the intestines has not been a marked feature although capsulatus was abundant. In other cases large amounts of indol have been formed. In such instances we may either have to do with a strain of capsulatus which is a good indol maker or with the symbiotic action of capsulatus and B. coli or some other intestinal micro-organism. I am inclined to believe that other bacteria than B. aerogenes capsulatus are generally concerned in putrefaction when indol formation is large in the gut.

We may consider now some of the conditions of the intestinal tract in which B. aerogenes capsulatus is present in excessive numbers and exerts pathological effects.

Even in early life the intestinal tract is not rarely the seat of capsulatus infection. Careless feeding apparently offers the conditions favorable for the temporary establishment of the organism, which manifests itself in irregularities of the bowels, diarrhœa, movements showing saccharo-butyric putrefaction, and ultimately anæmia. In Case XIV we have an example of capsulatus infection in a young child fed on cow's milk in which a severe form of anæmia was developed in the course of a few months. Tissier[1] has described an intestinal infection in breast-fed and bottle-fed children, which occurs especially in warm

[1] *Ann. de l'Inst. Pasteur*, xix, p. 273, 1905.

weather. The disease is attributed by him to the organism known to the French as B. perfringens. It appears, however, that B. perfringens is identical with B. aerogenes capsulatus. It appears highly probable that the diarrhœas of some breast-fed infants are to be referred to capsulatus infection.

From normal infancy and child-life to the time of adolescence the movements are remarkably free from B. aerogenes capsulatus. They can generally be isolated even from such material, but only in such small numbers as to be probably insignificant physiologically and pathologically.[1]

In this connection it should not be forgotten that Klein isolated from the feces in an outbreak of diarrhœa an organism which very probably is identical with B. aerogenes capsulatus. This organism was given the name B. enteritidis sporogenes. The position of the organism described by Klein is open to some suspicion because while it is stated by him to produce gas and butyric acid in the very characteristic manner observed by Welch and Nuttall for B. aerogenes capsulatus it differs from the latter in having motility and apparently in sporulating much more readily. Cultures sent by Dr. Klein to Professor Welch contained bacilli which agreed in every detail with pure cultures of B. aerogenes capsulatus.[2] As, however, the feces sometimes contain bacteria having the morphology of B. aerogenes capsulatus but differing from this organism in forming spores much more readily it is possible that Klein was in reality dealing with impure cultures of B. aerogenes capsulatus.

In early adult life B. aerogenes capsulatus is often present in the intestine in considerable numbers, even when health is apparently good. I have not, however, observed any instances in which it was very prominent among the fecal bacteria without some signs of intestinal disturbance, with evidence of excessive intestinal putrefaction or more obvious symptoms, such as diarrhœa and flatulence.[3]

[1] Hirshberg, working with Professor Welch, found the organisms to be widely distributed in the intestines of animals.

[2] See *Manual of Bacteriology* by Muir and Ritchie, American Edition, 1904, p. 354.

[3] In one instance which has come under observation the feces contained abundantly an organism appearing to be B. aerogenes capsulatus, although the subject was not ill in any ordinary sense. The subject was a woman

There are many persons who have from time to time slight diarrhœal disorder associated with a great temporary abundance of B. aerogenes capsulatus and streptococci in the stools, but who keep in fair health for years in spite of these recurrent seizures. These persons are apt to develop a moderate grade of anæmia and an intolerance for vegetable acids and carbohydrates. The intolerance for carbohydrates is probably connected with the presence of capsulatus in the small intestine. We know that this organism grows readily on carbohydrate media and it is likely that it multiplies, in many persons, in the small intestine, where carbohydrates have been freely eaten. The descent of the organism to the colon in large numbers is followed by active saccharobutyric putrefaction and its consequences. I have noticed capsulatus growing in long threads in the feces of a person who had diarrhœa following an excessive carbohydrate meal. This thread-like development may also be observed in the livers of dead rabbits which have been incubated after intravenous infusion of capsulatus In both cases the thread-form is attributable to an abundance of carbohydrate pabulum.

It is highly probable that there are few persons who do not from time to time suffer intestinal derangements connected with over-multiplication of organisms of the capsulatus type and that normal bacterial conditions are in most cases quickly re-established, after the subsidence of these seizures. But there may also come a time in the life-history of an individual who has had these seizures repeatedly when the capsulatus organism fixes itself persistently in the intestinal tract and assumes a pseudo-parasitic instead of a saprophytic relation to the host. The capsulatus organism is then found to be abundantly and regularly present in the movements. Coincidently there is a decline

eighty-five years of age in a remarkably good state of preservation. She was distinctly feeble and felt herself growing gradually feebler, but was only moderately anæmic. In this case there was an inclination to constipation and the urine and feces were uncommonly free from putrefactive products. It should be noted, however, that this comparatively good condition was maintained only by the utmost moderation in diet, the avoidance of sweets, highly seasoned food, etc. Slight indiscretions were regularly followed by diarrhœa and prostration. Micro-organisms of the B. coli type were abundant and well preserved in the fecal field, and gas production was moderate on the part of the fecal flora.

in the general health—often a very slow decline—characterized by loss of strength, slight anæmia and often, though not necessarily, loss in weight. Evidences of excessive putrefaction are commonly, though not invariably associated with this slow decline in physical status—a decline which at this period of the process may be temporarily or indefinitely checked by suitable measures. In more extreme instances the process of capsulatus decomposition within the intestine attains a high degree of intensity, partly on account of the large numbers of the organism, partly, perhaps, owing to the development (or original possession) of more than usually marked pathogenic properties on the part of the micro-organism, partly, perhaps, because a large tract of the intestine is implicated.[1] These extreme instances are marked by the development of a high grade of anæmia. Indications are not wanting that quantitative alterations in the blood are present, often for a long time before the hæmoglobin falls greatly or the red cells are much reduced in numbers. Ultimately there develops the blood picture of a severe secondary anæmia or the picture of pernicious anæmia.

In those cases where the feces contain regularly very large numbers of B. aerogenes capsulatus and few living organisms of the B. coli type, it is not yet clear which is the order of events in the establishment of this abnormal relationship—whether the elimination of B. coli tends to overgrowth of the gas bacillus or whether a great increase in the gas bacilli leads to the suppression of the colon bacillus. It seems probable that the dominance of the one type necessitates conditions which favor the restriction of the other. For the strictly anaerobic condition which is necessary to the growth of the gas bacillus is in itself inimical to the best development of B. coli. On the other hand B. coli thrives under fairly aerobic conditions which in themselves quite check the gas bacillus, although it is conceivable that under some circumstances B. coli may favor the growth of the gas bacillus by the appropriation of oxygen.

[1] Of the conditions of capsulatus activity in the small intestine we at present know nothing, but the suspicion seems not unwarrantable that the organism under some conditions multiplies actively even in the upper part of the small intestine. The chief evidence of this is the presence in the small intestine of micro-organisms morphologically resembling B. aerogenes capsulatus in some persons dying from intestinal disorders.

According to the view here put forward pernicious anæmias and secondary anæmias are often closely connected with a toxic-putrefactive process in the intestine initiated by a gas-forming organism of wide distribution—B. aerogenes capsulatus. The strongest arguments in favor of a causal relationship in some cases of anæmia are (1) the abundance of this organism in the feces of such patients as compared with their moderate occurrence in the feces of normal individuals; (2) the gradual reduction in the number of these organisms as the clinical conditions undergo improvement; and (3) the ability of the B. aerogenes capsulatus to make a hæmolytic substance or substances. Considerable work remains to be done to establish the nature of the hæmolytic action of the capsulatus organism and it also remains to be seen whether it is possible by means of capsulatus products to induce extreme forms of anæmia in animals closely related to man. In the absence of the latter evidence the dependence of anæmia on capsulatus infection is an hypothesis rather than an established fact, but it is one which is put forward as already possessing a high degree of probability. The fact that streptoccocus infection is sometimes associated prominently with capsulatus infection in advanced anæmias does not, I think, weaken the significance of the association of anæmia and capsulatus infection.[1] But it is to be distinctly emphasized that the claim here made is not that severe anæmia is an invariable or exclusive result of capsulatus infection of the intestine, but rather that it is an important incident of certain extreme cases of chronic capsulatus infections.

It is possible that an important factor in determining the nature of the pathological action exerted by B. aerogenes capsu-

[1] There certainly are instances of streptococcus infection of the intestine (sometimes of oral origin) in which there develops a moderate or pronounced anæmia which may reasonably be referred to the influence of this infection, since evidence is wanting of the presence of other pathogenic micro-organisms in significant numbers. There are also instances in which we have to deal with a combination of streptococcus and capsulatus infection. Among our cases of anæmia there are a number in which streptococci were regularly very abundant in the microscopical fields, and cultures from such cases have shown streptococcus to be dominant. The significance of combined streptococcus and capsulatus infection of the intestine is not yet clear. The ability of certain streptococci to produce

latus in the intestine is the state of representation of the B. coli group. I am inclined to attach much importance to the falling out of the B. coli group (as indicated by restricted gas production and the appearance of the microscopical fields) in association with the presence of B. aerogenes capsulatus in greatly increased numbers, since such a condition points to the withdrawal of what is probably a protective action of the utmost physiological value.

There is one criticism of the hypothesis here advanced which it seems desirable to forestall. What evidence is there, it may be asked, to show that the presence of excessive numbers of B. aerogenes capsulatus in the feces is not the result of anæmic states rather than their cause? May it not be possible that conditions of anæmia so alter the digestive secretions as to favor the development of organisms of the capsulatus type and to depress the multiplication of the representatives of the B. coli group? The only answer to these questions lies in those clinical observations which show that a serious disorder of digestion has antedated the appearance of anæmia. In three of our cases (X, XII, and XVI) the history is quite explicit on this point. Moreover in each of these cases there was a subsidence of the capsulatus infection before recovery from anæmia occurred. Finally, we have had under observation cases of secondary anæmia from other well-defined and recognized causes of anæmia, in which capsulatus organisms were present only in moderate numbers and in which putrefactive disorders were absent. These facts do not perhaps suffice to prove that capsulatus infection is the cause rather than the result of anæmic states, but they go far to render probable the causal relationship which has been advocated, while at the same time they make it very improbable that anæmia is in itself a cause of the dominance of the capsulatus type of organisms in the digestive tract.

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hæmolytic substances suggests that these organisms may be factors in the production of anæmia.

Dr. Rettger tells me that in two cases of pernicious anæmia studied by him, the dominant micro-organisms in the plates were streptococci. In these cases no growth of B. coli was obtained. Strict anaerobes were also absent from the plates.

Hunter (*loc. cit.*, pp. 236 and 242) attaches much importance to the passage into the stomach of streptococci and other pyogenic micro-organisms derived from the diseased mouth. His observations on this subject apparently did not include the bacteriological study of the feces.

It seems not improbable, however, that conditions which, like gastric carcinoma, weaken the defence of the digestive tract against pathogenic bacteria by the withdrawal of the acid secretion of the gastric juice, pave the way for capsulatus infection, which, when established, may heighten the anæmic cachexia referable to the carcinomatous growth. There are some facts which point in this direction but which need not be further discussed here.

I take especial satisfaction in thanking my friend and colleague, Professor Theobald Smith for the many helpful suggestions he has made in the course of this work regarding questions of bacteriological technique and of micro-organic biology. I desire also to acknowledge the important aid I have received from Miss M. L. Foster and Dr. Helen Baldwin in connection with the analytical work contained in this paper.

CONCLUSIONS.

The following is a summary of the conclusions reached in the course of the research presented in the preceding pages:

1. The human intestine of normal adults and of normal children frequently holds small numbers (relatively to other bacteria) of the anaerobic, difficultly spore-forming, gas-making bacillus described by Welch and Nuttall as B. aerogenes capsulatus.

2. In some conditions of disease the intestinal tract shows the presence of anaerobic, difficultly spore-forming, gas-making bacilli, identical with B. aerogenes capsulatus, in greatly increased numbers.

3. This increase in the number of B. aerogenes capsulatus in the intestine may be temporary or it may be a permanent condition and may constitute a definite infection of the gastroenteric tract.

4. The presence of B. aerogenes capsulatus in excessive numbers in the feces is determinable by plating in sugar-blood agar, by the inoculation of sterilized milk in fermentation tubes, by the procedure of intravenous infusion of fecal suspensions into rabbits and subsequent incubation, and by the microscopical examination of the Gram-stained fecal fields.

5. The infection of the intestinal tract with B. aerogenes capsulatus leads to varying clinical results according to the intensity of infection, its duration and its bacteriological associations. Moderate numbers of the bacilli administered to monkeys cause only slight diarrhœa with increase of the capsulatus organisms in the stools. There are frequent diarrhœal conditions in breast-fed and bottle-fed children which seem clearly referable to B. aerogenes capsulatus. Many temporary intestinal derangements in adults are associated with a temporary increase in B. aerogenes capsulatus. In children capsulatus infection may lead to the development of extreme anæmia with general œdema.

6. In many acute and subacute capsulatus infections of the intestine, the living micro-organisms of the B. coli type in the *feces are much reduced in number. This condition of colon* bacillus sparsity is shown (a) by the appearance of the Gram-stained fecal fields; (b) by the diminished production of gas in fermentation tubes; (c) by the failure of the sediments in fermentation tubes to show a satisfactory growth of organisms of the B. coli type on sugar bouillon; and (d) by the small number of colonies found on gelatin plates or their entire absence.

7. Chronic conditions are not rare in which the feces show a condition of colon bacillus scarcity associated with a marked increase in the numbers of B. aerogenes capsulatus. The persons in whom these bacterial conditions have been persistent almost always are at least moderately anæmic, *i. e.*, show some fall in hæmoglobin and indications of a diminished volume of blood.

8. B. aerogenes capsulatus is prominently characterized by the ability to induce a characteristic type of putrefactive decomposition which may be designated *saccharo-butyric putrefaction*. Among the chief products of saccharo-butyric putrefaction are carbon dioxide, hydrogen, butyric acid, and ammonia. This putrefaction may occur on proteid media containing very little carbohydrate material.

9. The excessive formation of gas and the consequent flatulence in many cases of capsulatus infection of the intestine. are referable to excessive saccharo-butyric putrefaction or fermentation.

10. The excess of higher volatile fatty acids, including

butyric, which is observed in many cases of capsulatus infection is referable to excessive saccharo-butyric putrefaction.

11. B. aerogenes capsulatus is an active hæmolysing agent. The hæmolysis induced by it *in vitro* can probably be referred in part, but only in part, to the formation of ammonium butyrate.

12. Many instances of "primary" pernicious anæmia and of secondary anæmia show pronounced indications of excessive saccharo-butyric putrefaction, but the fecal and urinary manifestations of this process differ considerably in different instances. An increase of indol in the feces and of indican in the urine are common, though not regular manifestations. A high grade of intestinal putrefaction with excessive formation of phenol and indol may occur in persons who show few capsulati in the feces.

13. There are instances of "primary" pernicious anæmia and others showing the blood changes of secondary anæmia (of undeterminable etiology), in which there is chronic infection of the intestinal tract with B. aerogenes capsulatus. The gas bacillus is in these cases the dominant spore-forming anaerobe. The representation of living micro-organisms of the B. coli type in the feces is usually much reduced in these cases.

14. In certain instances of advanced anæmia it has been observed that as the blood picture and the general conditions improve there is a distinct reduction in the numbers of B. aerogenes capsulatus in the feces together with a better representation of the B. coli group. With these altered bacterial conditions there is usually an increase in the capacity for gas production by the mixed fecal flora.

15. The close association between certain anæmias and capsulatus infection of the gastro-enteric tract creates a presumption that this infection stands in a causative relation to these anæmias although experimental evidence of such a relation has not yet been obtained.

16. In some of the anæmias associated with capsulatus infection, large numbers of streptococci are found in the feces. The significance of this mixed infection is not yet clear.[1]

[1] The following practical considerations respecting infection through the agency of food deserve mention:

An important practical measure in all cases of capsulatus infection is the avoidance of food containing B. aerogenes capsulatus. As an organism

17. It sometimes happens that the numbers of B. aerogenes capsulatus in the feces undergo such a great decrease in the course of treatment that the microscopical fecal fields present a nearly normal appearance with respect to this organism. Therefore the failure to find B. aerogenes capsulatus in large excess after a patient has for some weeks or months had the benefit of treatment by diet and rest, does not necessarily exclude a preceding infection.

ABSTRACT OF CLINICAL NOTES TO CASES REFERRED TO IN THE FOREGOING PAPER.

CASE I. Presbyterian Hospital, service of Prof. James. Male, æt. about 45. Increasing weakness of legs before entry to hospital, some looseness of bowels, occasional vomiting. Hb. = 30 per cent.; red blood cells, 1,248,000; leucocytes, 5200; normoblasts, 1 per cent.; megaloblasts, 3 per cent.; many megalocytes; marked poikilocytosis. Later examinations with essentially same results.

CASE II. New York Hospital, service of Prof. Lambert. Woman æt. 58. Frequent diarrhœa with increasing weakness for past year with loss of 20 lbs. weight. Complains of weakness. Hb. = 35 per cent.; red blood cells, 1,440,000; red cells show marked granular degeneration. Many normoblasts, no typical megaloblasts, mitosis in normoblasts, Red cells larger than normal. No typical megalocytes. Later examinations with similar results.

CASE III. French Hospital, service of Dr. N. B. Potter. Woman æt. 65. Malaria 15 years ago in Smyrna, but well until 1 year ago. Since then, gradual loss of strength and color. Is now thin, toothless, and very pale. Hb. = 40 per cent.; red blood cells, 1,776,000; leucocytes, 5000; megaloblasts, 6, and normoblasts, 7 to 100 leucocytes. Patient shows little tendency to improvement as result of usual treatment for anæmia.

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identical with B. aerogenes capsulatus or very closely related to it (Grassberger and Schattenfroh's granulo-bacillus saccharo-butyricus immobilis liquefaciens) is often found in milk, it is especially important that attention should be given to the anaerobic micro-organisms of the milk and milk products employed as food. As sewage generally contains large numbers of B. aerogenes capsulatus, oysters exposed to sewage may become a source of intestinal disease from this micro-organism.

The official examination of milk by Health Boards might advantageously include the simpler tests (especially the action on milk resulting in "stormy fermentation") for the presence of saccharo-butyric putrefactive, spore-forming micro-organisms.

Case IV. St. Luke's Hospital, service of Dr. Spalding. First admitted 2 years ago with pneumonia and anæmia. Hb. = 35 per cent.; red blood cells, 1,328,000; megaloblasts found. No free hydrochloric acid. Since then has been very weak, with much emesis, anorexia, diarrhœal and sometimes bloody stools and cold extremities. Typical result of one of several recent examinations: Hb. = 41 per cent.; red blood cells, 1,032,000; leucocytes, 4000; polymorphonuclears, 14 per cent.; lymphocytes, 26 per cent.; normoblasts; aniso- and poikilocytosis.

Case V. Presbyterian Hospital, service of Dr. Thacher. Male, æt. 64. For 6 months dyspnœa and palpitation with loss in strength and weight and increasing pallor. Three weeks before admission, severe seizure of diarrhœa following retching after severe exertion. No teeth except two in lower jaw. Slight œdema over whole body. Slight rise in temperature. Repeated examinations of blood; typical one as follows: Hb. = 30 per cent.; red blood cells, 1,500,000; leucocytes, 3300; red cells show pallor; many myelocytes; marked poikilocytosis, polychromasia, and basophile granulation; normoblasts and megaloblasts present.

Case VI. New York Hospital, service of Dr. Lambert. Woman, æt. 49. Gradually increasing dyspnœa, palpitation, weakness, and headache. Nothing in previous history to give clue to etiological factor. Typical examination, Hb. = 33 per cent.; red blood cells, 976,000; leucocytes, 7400; many oval reds, moderate poikilocytosis; typical megalocytes. Normoblasts found in some examinations.

Case VII. Presbyterian Hospital, service of Dr. Thacher. Male, æt. 40. Small and thin. Pulmonary and testicular tuberculosis, evening temperature. Bowels irregular, movements soft. Hb. = 28 per cent.; red blood cells, 1,288,000; leucocytes, 9000; polymorphonuclears, 50 per cent.; transitionals, 7 per cent.; large mononuclears, 6 per cent.; lymphocytes, 27 per cent.; megalocytes and megaloblasts. On light diet, lavage of colon, and arsenic patient gradually improved. Three months after admission, Hb. = 62 per cent.; red blood cells, 3,792,000; leucocytes, 7900. Patient's color and strength steadily improving.

Note. This is one of the cases in which improvement coincided with a great diminution of B. aerogenes capsulatus in the feces and with a return of a good representation of bacteria of the B. coli type.

Case VIII. Roosevelt Hospital, service of Prof. James. Male, æt. 38. Typhoid fever 7 years ago; 2½ years ago, jaundice for one year. Since jaundice, weakness, dyspnœa, and anorexia. Sallow, fairly nourished, two carious molars. Hb. = 30 per cent.; red blood cells, 1,768,000; leucocytes, 6300; myelocytes, megaloblasts, normoblasts, poikilocytes, Other examinations with similar results.

Case IX. Roosevelt Hospital, service of Prof. James. Male, æt. 63. Weakness, anorexia, pallor, and dyspnœa for a year. Etiological factors

not discovered. Repeated examinations of blood of which following is typical: Hb. = 45 per cent.; red blood cells, 1,656,000; leucocytes, 13,000; megaloblasts, megalocytes, microcytes, macrocytes, polychromatophilia, poikilocytosis. Blaud's pills, Fowler's solution, colon irrigation, carnogen. Hb. fell to 30 per cent., red blood cells to 876,000. Death 2 months after admission.

CASE X. St. Luke's Hospital, service of Dr. Spalding. Male, æt. 30. Seven years ago became very weak and anæmic. Much digestive disturbance, alternating constipation and diarrhœa. Nausea, frequent vomiting of bile. Four years ago, Hb. = 25 per cent.; red blood cells, 928,000; two years ago, after temporary improvement, Hb. fell to 34 per cent.; red blood cells, 1,572,000; megaloblasts. No free hydrochloric acid. Temperature ranged from 101° to 102.5° F. On certain occasions megaloblasts abundant. After gradual improvement, was able to work 10 hours daily as switchman. One year ago entered hospital with all signs of pernicious anæmia. For a time patient's blood condition retrograded until Hb. was 25 per cent.; red blood cells, 660,000. After systematic colon irrigation, slow but steady improvement. After 3 months, Hb. = 90 per cent.; red blood cells, 3,086,000. Discharged greatly improved and able to walk several miles without fatigue.

Note. The feces of this patient were repeatedly examined for anaerobic micro-organisms. During the period of most pronounced anæmia and weakness, B. aerogenes capsulatus was very abundant in the feces, as was also indol. At the height of convalescence there was noted repeatedly a marked decline in these micro-organisms and in the putrefactive products, in the feces. Marked increase in numbers of colon bacilli in feces during period of improvement.

CASE XI. City Hospital, service of Dr. Ransom. Female about 50 years of age. In ward several months for weakness and anæmia. Digestive history not carefully recorded. Hb. = 20 per cent.; red blood cells about 1,000,000; leucocytes, 9000; no megaloblasts; no nucleated red cells, no morphological changes except marked deformity of red cells. At autopsy nothing was found to account for the profound anæmia.

CASE XII. Notes furnished by Dr. Fred. Shattuck of Boston. Male, æt. 73. Always robust until about 8 years ago. At that time a digestive disturbance which resulted in loss of strength. Apparently full recovery. Nearly one year ago a severe diarrhœal seizure lasting two weeks and causing confinement to bed. At this time, slightly anæmic. Eight months ago had become weak and had lost weight, but continued at business. Bowels irregular with diarrhœal tendency. Somewhat later was confined to bed. About 6 months ago, red blood cells numbered 1,800,000; megaloblasts and normoblasts observed. Gradual improvement set in 3 months ago. Hb. = 82 per cent.; red blood cells, 4,010,000; no blasts. Patient gained steadily.

Note. Six months ago feces contained B. aerogenes capsulatus in abundance (relatively to B. coli). During period of improvement numbers of B. aerogenes capsulatus showed distinct falling off; quite recently the number has again increased to a point far above the normal.

CASE XIII. Vanderbilt Clinic, service of Dr. Patterson. Male, æt. about 40. Gradual development of anæmia and weakness during past year. Blood examination gives indications of pernicious type of anæmia.

CASE XIV. Nursery and Child's Hospital, service of Dr. Lyon. Girl, æt. 3 years. Enteritis during summers of 1904 and 1905. Abdomen large, spleen reaches middle line; liver 2 inches below ribs in nipple line; lymph nodes in axilla and neck moderately enlarged. Typical blood examination showed Hb. = 25 per cent.; red blood cells, 1,250,000; slight lymphocytosis; nucleated red blood cells; normoblasts, megaloblasts, some mitotic figures. Child was on a general diet.

The diagnosis in this case was left in doubt. The bacterial and chemical examination of the feces and urine showed nothing which could be regarded as accounting for the observed anæmia.

CASE XV. Notes furnished by Dr. N. B. Potter. Male, æt. 45. For about 1 year, loss of weight, weakness, mental depression, and anæmia. Various derangements of intestinal digestion. No free hydrochloric acid Hb. = 35 per cent.; red blood cells, about 2,000,000. No improvement under influence of rest, iron, and arsenic. Diagnosis in doubt; carcinoma of stomach considered; also early stage of primary pernicious anæmia.

CASE XVI. Babies' Hospital, service of Dr. L. E. Holt. Female child, æt. 20 months. For several weeks, intestinal catarrh and diarrhœa Loss of weight, anæmia, very peevish, general œdema, casts in urine. By careful feeding, intestinal conditions were rapidly improved, œdema subsided, weight increased, and nervous symptoms subsided. Dec. 1, 1905, weight, 11 lbs., 13 oz.; Dec. 14, 13 lbs., 4 oz.

The blood history is as follows:

Dec. 1, 1905: Hb. = 35 per cent.; red blood cells, 1,798,400; leucocytes, 8000; polymorphonuclears, 38 per cent.; mononuclears, 58 per cent. large mononuclears, 3 per cent.; eosinophiles, 1; nucleated reds, about 2 per cent.; a good deal of basophilic degeneration in the reds.

Dec. 9, 1905: Hb. = 50 per cent.; red cells, 2,390,000; leucocytes, 15 per cent.; polymorphonuclears, 39 per cent.; small mononuclears, 50 per cent.; transitionals, 10 per cent.; some eosinophiles, numerous normoblasts, a few megaloblasts. Abnormal degeneration of red cells

Dec. 16, 1905: Hb. = 70 per cent.; red cells, 3,667,200; leucocytes. 12,000; polymorphonuclears, 51.6 per cent.; small mononuclears, 35 per cent.; large mononuclears, 8.25 per cent.; transitionals, 1.6 per cent.; eosinophiles, 1 per cent.; basophiles, 2.6 per cent.; nucleated reds, 1.

Dec. 23, 1905: Hb. = 75 per cent

Note. At onset, the stools contained very large numbers of B. aerogenes capsulatus; B. coli were present in very small numbers. As improvement occurred, there was a return of B. coli and B. aerogenes capsulatus was greatly reduced in numbers.

Case XVII. Patient of Dr. Skinner, New Haven. Notes furnished by Prof. L. B. Mendel and Dr. L. F. Rettger. Male, æt. about 30. Formerly a painter. Is emaciated and appears wax-like. Hb. = about 20 per cent.; red blood cells, 380,000. Numerous nucleated red cells and megaloblasts. Treated by exposure to dry baking temperature. Rise to 2,600,000 red cells in short time.

STUDIES ON PUTREFACTION.[1]

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(Received for publication, May 18, 1906.)

The study of putrefaction has received a new impetus in recent years. While its chemistry has been given considerable attention by such men as Baumann, Brieger, Nencki, Salkowski, and numerous other investigators, much remained and still remains to be learned regarding the relation of various bacteria to putrefactive processes. It is an accepted fact that all real putrefaction is due to bacteria; but what particular micro-organisms are responsible for such decomposition of proteids, and the conditions which influence the nature and rate of decomposition, are matters that require much further study.

Pasteur claimed that putrefaction could occur only in the absence of free or atmospheric oxygen. This view was later supported by the observations of other investigators, particularly Nencki, Bovet, Kerry, and Bienstock. In addition to this, Bienstock claimed that putrefaction is the work of a particular class of bacteria, the anaerobes. On the other hand, investigations have been made which appeared to show that not only the obligatory anaerobes, but also the facultative aerobes possessed the power of inaugurating putrefactive changes in proteids. It is perhaps safe to say that this view is held by the majority of biologists and chemists of to-day.

In a previous article on bacterial decomposition of proteids the writer[2] also adhered firmly to the last mentioned view, which was supported apparently by certain observations which he had made regarding the chemical products of B. coli communis and B. lactis aerogenes, in a culture medium consisting of coagulated egg-white and lean beef.

[1] The investigation upon which this paper is based was carried on with the aid of appropriations from the Rockefeller Institute for Medical Research.

[2] Rettger, *Amer. Journ. of Physiol.*, viii, p. 284, 1903.

Since the publication of that article, I have been engaged for almost two years in a further study of putrefaction; but this time with far different results. It required fully a year's constant work, and the preparation and final analyses of some fifty culture flasks inoculated with different facultative aerobes, particularly members of the colon bacillus group, to arrive at the later conclusion; namely, that the ordinary facultative aerobes cannot initiate putrefactive changes in the so-called egg-meat mixture.

The chief obstacle in the final solution of this question was the difficulty of precluding certain contamination forms (anaerobes) from the flasks. Although these intruders were spore-producers, they for a long time regularly escaped my notice. At the time of the examinations the spores must have been free, and obscured by the granular nature of the decomposition mixture; hence my failure to detect them. As soon as the contamination forms were discovered, every precaution was taken to exclude them. The laboratory floors were newly oiled; table tops, shelves, etc., were washed with a strong solution of corrosive sublimate; and finally, the flasks, stoppers, delivery tubes, etc., were thoroughly sterilized.

The result of all this was that the egg-meat mixtures which were thereafter inoculated with pure cultures of B. coli and B. lactis aerogenes underwent no decomposition. Not one of the fourteen coli strains (isolated from twelve different samples of stools) caused any visible change within the flasks, even after three or four weeks of incubation at 37° C.

In his study of phosphorus-containing bacterial products, Stich[1] employed B. coli to bring about decomposition and assumed that the putrefactive changes which he obtained were altogether due to this organism.

Emmerling[2] obtained marked putrefactive changes in sugar-free wheat bran with what he thought was a pure culture of Proteus vulgaris. In fact, in the literature this organism is quite commonly spoken of as a putrefactive organism. Emmerling[3] also claims to have brought about extensive putrefaction

[1] Stich, *Chem. Centralbl.*, i, p. 1137, 1900.

[2] Emmerling, *Ber. d. deutsch. chem. Gesellsch.*, xxix, pp. 2721–2726, 1896.

[3] Emmerling, *ibid.*, xxx, p. 1863, 1897.

of egg albumin and blood fibrin with Staphyloccocus pyogenes, and Streptococcus pyogenes, respectively. As in my own experiments, it is quite probable that the above proteid decompositions were caused by one or more of the almost omnipresent anaerobes which in an unforeseen way made their entrance into the media.

In the investigations of Buchner, Leber, Fränkel, Brieger, and Lübert[1] on the decomposition products of pus cocci, the results were to a large extent contradictory. Brieger found that albumins were attacked only with the greatest difficulty.

The most important contributions to this subject in recent years are, beyond a doubt, those of Bienstock.[2] His conclusion may be summed up in these words: "There is no real putrefaction without the action of obligate anaerobes." By the term "putrefaction" he meant, as is now understood generally, a bacterial decomposition of albuminous matter which is accompanied by the formation or elimination of foul-smelling substances, the so-called "Fäulnissprodukte."

While his statements may appear somewhat extravagant, I believe it may be safely said that none of the known facultative anaerobes has the ability to produce genuine putrefaction; and, as anaerobic conditions are necessary, the obligate aerobes can take no important part in such a process.

In the general destruction of waste organic matter the initial decomposition is caused by the anaerobes which appear to be everywhere present. If a tube of sterile bouillon or any other culture medium be inoculated with but a grain of garden soil, dirt from the street, scrapings from old harness, etc., it will invariably undergo putrefaction, under anaerobic conditions. The commonest, or at least best known, of these anaerobes are the Bacillus putrificus of Bienstock (B. cadaveris of Klein), the bacillus of malignant œdema and the bacillus of symptomatic anthrax.

B. putrificus is of particular interest. It was first discovered and described by Bienstock.[3] He found it in great abu ance

[1] See Emmerling, *loc. cit.*, p. 2724.

[2] Bienstock, *Arch. f. Hyg.*, xxxvi, pp. 335–389, 1899; *ibid.*, xix, pp. 390–427, 1901.

[3] Bienstock, *ibid.*, xxxvi, pp. 335–389, 1899.

in ordinary street dirt. The same bacillus was observed by Klein[1] in putrefying cadavers, and therefore called by him B. cadaveris. He regarded it as being wholly responsible for the putrefactive decomposition of cadavers.

The B. putrificus is a long, slender organism which grows very rapidly under strictly anaerobic conditions and is actively motile. It produces spores, as a rule, in about three or four days, at 37° C. The spores are large and terminal, and give the organism the characteristic drumstick appearance. Motility persists long after spores are produced. In its morphology and in most of its cultural characteristics it closely resembles the tetanus bacillus, and is, no doubt, frequently mistaken for it. The B. putrificus does not, however, have pathogenic properties. The spores of B. putrificus are very resistant to heat, and are therefore often responsible for the putrefaction of culture media after ineffectual sterilization.

I have succeeded in finding and identifying the B. putrificus in numerous samples of soil taken from gardens, barn-yards, wood piles and the street, in dust about the laboratories and elsewhere, and in beef muscle on various occasions. It is very difficult, however, to isolate this organism, as Bienstock also informs us. This can be done, nevertheless, by alternately heating at 80° C. for 10 minutes and cultivating under anaerobic conditions, several times each, and finally pouring sugar-agar plates. The colonies resemble those of B. tetani, to a large extent, though as a rule they are coarser.

The bacillus of malignant œdema and the bacillus of symptomatic anthrax likewise occur in soil, street dirt, and in the cadavers of certain animals. It is no difficult matter to obtain the bacillus of malignant œdema in pure culture from the muscles of a guinea-pig soon after death. We have also found it to be present frequently on the surface of fruits, particularly the banana.

EXPERIMENTAL.

The work recorded in this paper has been carried on largely with various strains of B. coli communis and B. lactis aerogenes

[1] Klein, *Centralbl. f. Bakt., etc.*, xxv, 1 Abt., pp. 278, 1899.

which were recently isolated from normal stools, and also those of pernicious anæmia patients. As strict anaerobes, different strains of B. putrificus, the bacillus of malignant œdema, and the bacillus of symptomatic anthrax were employed. The medium which was used was the "egg meat" mixture, and the decomposition tests were made in liter flasks and in large test-tubes. This particular medium was chosen primarily on account of the small cost and ease of preparing it; and secondly, because, as native proteids, meat and eggs form a large part of a person's diet. This is of especial importance as relating to the subject of intestinal putrefaction.

Preparation of Egg-Meat Mixture.—A. One half pound of lean chopped beef is stirred up in 250 c.c. of water, and after neutralizing the meat acids with sodium carbonate, the mixture is heated in an Arnold sterilizer for 30 minutes, with occasional stirring. It is then set away in a cold place for several hours after which the fatty scum is removed.

B. The whites of three eggs are mixed with 250 c.c. of water, and after neutralizing, the albumin is coagulated, with occasional stirring, by heating in the Arnold sterilizer for 30 minutes.

A and B are then mixed and introduced into a liter flask, along with 2.5 grams (0.5 per cent.) of powdered calcium carbonate. The flask is plugged with cotton and sterilized for 30 minutes, at 110–112° C. (autoclave).

Cultivation of Bacteria.—After inoculating the flasks with the desired organism, the old plug is replaced by a dry, sterile cotton plug having a glass tube through the centre. The plug and tube are pushed down into the neck of the flask, and a rubber stopper provided with two bent glass tubes (inlet and outlet) is inserted in the mouth of the flask, after connecting the inner tube with the outlet tube by means of a rubber joint. Hydrogen gas is then rapidly passed through the flask from a Kipp's generator, to remove all oxygen. In order to free the hydrogen gas from arsenic, it is passed through a strong solution of potassium permanganate. The flask is then placed in the incubator and connected with a mercury bottle for relieving pressure, and at the same time preventing air from entering the flask.

Methods of Analysis.[1]—Two different portions of the decomposition material are employed; one for the study of indol, skatol, phenols, aromatic oxy-acids, skatol-carbonic acid, tyrosin, leucin, albumoses and peptone, and tryptophan—I; and the other for the determination of hydrogen sulphide and mercaptan—II.

I. INDOL, SKATOL, PHENOLS, ETC.—Two hundred cubic centimeters of the well-mixed putrefaction material are diluted with an equal volume of water, and after the addition of 5 c.c. of dilute sulphuric acid, are distilled with steam until 400 c.c. of distillate are obtained. The distillate is made alkaline with potassium hydrate, and again distilled with steam until 250 c.c. of liquid have collected; indol and skatol are in this distillate—(A). The last distillation residue is saturated with carbon dioxide gas and distilled (steam) until 250 c.c. are obtained; phenols are present in this distillate—(B).

For the determination of indol, definite quantities of Distillate A are diluted with known quantities of water until the dilution is such that the color reaction[2] obtained with nitric acid is barely, but distinctly, perceptible. The amount of dilution required is an index to the approximate quantity of indol[3] present. A similar test is made for skatol in the same distillate, only instead of using nitric acid, the color reaction is obtained with a few drops of *concentrated sulphuric or hydrochloric acid*, in the absence of nitrous acid.

For the estimation of phenols, Distillate B (supposed to be absolutely free from indol) is diluted in the same manner as A, if necessary, and the *color reaction obtained* with Millon's reagent.

AROMATIC OXY-ACIDS AND SKATOL-CARBONIC ACID.—The liquid

[1] These methods differ but little from those described in my previous paper. While their results cannot be claimed to be at all accurate, they are of much value in comparative studies of bacterial products.

[2] For color reactions of indol, skatol, aromatic oxy-acids, and skatol-carbonic acid, see Salkowski's *Praktikum der physiologischen und pathologischen Chemie*, 1900.

[3] Herter's naphthaquinone reaction was also employed on several occasions, and proved to be a very delicate and convenient reaction. Minute traces even of indol are indicated by it when the nitric-acid test has proven negative. See Herter, this Journal, i, p. 257, 1906.

residue remaining after the first distillation is filtered, concentrated to small volume and again filtered. It is then extracted with ether, in order to remove the aromatic oxy-acids and skatol-carbonic acid. The combined extracts are filtered and the ether removed by evaporation. The oily residue is extracted with warm water and the solution filtered. The combined extracts should equal 250 c.c. If necessary, a second extraction is made with the same quantity of water. Definite quantities of the 250 c.c. solution are examined for aromatic oxy-acids and skatol-carbonic acid in the same manner as was done for indol. The color reaction for aromatic oxy-acids is obtained with Millon's reagent, and that for skatol-carbonic acid with ferric chloride and dilute hydrochloric acid. (See Salkowski's *Praktikum.*)

TYROSIN, LEUCIN, ALBUMOSE, PEPTONE AND TRYPTOPHAN.—The liquid residue remaining after the ether extraction of aromatic oxy-acids and skatol-carbonic acid is concentrated to the point of crystallization, and after cooling the crystalline mass is examined for tyrosin and leucin. A second crop of crystals is obtained in the mother liquor. After removal of the tyrosin and leucin, the syrup is treated with four or five volumes of 95 per cent. alcohol; albumoses and peptone are thrown down, along with certain salts. The precipitate is washed with alcohol, dissolved in water, and the solution tested for albumoses and peptone by the usual reagents, particularly the biuret reaction. For the isolation and detection of tryptophan, the alcoholic filtrate and washings from the last precipitate are concentrated, acidified with acetic acid, and treated with 5 or 6 c.c. of bromine water. Tryptophan is precipitated as a purple bromine compound[1] which dissolves in alcohol, imparting the characteristic color to the solution.

II. HYDROGEN SULPHIDE AND MERCAPTAN. *a. Precipitation with mercuric cyanide.*—An equal volume of water is added to 200 c.c. of the decomposition material, in a 500 c.c. flask. The flask is connected, on the one hand, with a small precipitating bottle containing a 3 per cent. solution of mercury cyanide, and on the other, with a wash bottle containing permanganate solution. The flask is then acidified with 4 to 5 grams of oxalic

[1] Kurajeff, *Zeitschr. f. physiol. Chem.*, xxxvi, p. 501, 1898–99.

acid and warmed for 2 to 3 hours between 30 and 40° C. During the warming a current of air is slowly drawn through the flask. Hydrogen sulphide comes over first, if present, and produces a black precipitate in the cyanide bottle. After a while the mercaptan is seen to come over by the yellow precipitate which it produces in the cyanide solution. By replacing the mercury cyanide with fresh solutions, the two precipitates may be obtained apart from each other.[1] They are washed, dried, and weighed as mercury sulphide and mercury mercaptid respectively.

b. Mercaptan-Isatin reaction.[2]—A small quantity of isatin is dissolved in concentrated sulphuric acid. On passing mercaptan through this solution it is colored grass green. The reaction is a delicate one, and it is in no way interfered with by other decomposition products. The method of procedure is somewhat similar to that of the cyanide method; the isatin solution is substituted for the cyanide, and drying tubes (calcium chloride) must be employed to prevent any moisture from entering the isatin solution.

RESULTS OF INVESTIGATION.

As has already been stated earlier in this paper, the more carefully conducted decomposition experiments with the colon bacillus and B. lactis aerogenes conclusively demonstrated that these organisms are absolutely unable to transform the egg-meat mixture, even during several weeks of incubation. The flasks remained free from offensive odors, and there were no other signs of decomposition. It seems that B. coli communis and B. lactis aerogenes have but slight destructive action on peptone even, for in my previous work[3] I have never observed any marked reduction in the amount of peptone present in the peptone bouillon; and further, the quantities of indol, aromatic oxy-acids, etc., were always small.

All other facultative aerobes which have been tested so far have likewise given negative results in the egg-meat mixture; namely, B. fekalis alkaliganes, Proteus vulgaris, B. pyocyaneus,

[1] Nencki and Sieber, *Monatsh. f. Chem.*, 1889, p. 526.

[2] Niemann, *Arch. f. Hyg.*, xix, p. 126, 1893; also Bauer, *Zeitschr. f. physiol. Chem.*, xxxv, p. 346, 1902.

[3] Rettger, *Amer. Journ. of Physiol.* viii, p. 284, 1903.

B. fluorescens (liquefying and non-liquefying), B. cloacæ, B. prodigiosus, Staphylococcus pyogenes (aureus and albus), Micrococcus cereus albus, and Streptococcus pyogenes; also mixtures of two or more of these organisms, as, for example, B. coli communis, M. cereus albus and Streptococcus pyogenes. In every instance there were no visible signs of decomposition. Bienstock obtained the same results with some twenty of the more common facultative aerobes. In his experiments he used sterile blood fibrin.

Blood fibrin and egg-meat mixture do fall an easy prey, however, to the obligate anaerobes—B. putrificus, the B. of malignant œdema and the B. of symptomatic anthrax, and in all probability to a number of other anaerobes of which little is as yet known.

After inoculation of sterile egg-meat mixtures with either of the above anaerobes, they regularly underwent rapid decomposition. In less than 24 hours (37° C.) the characteristic putrefactive odor was already quite pronounced. By the third or fourth day there was considerable reduction in the amount of solid matter. This reduction continued until by the end of the seventh or eighth day only a small residue remained in the flasks, as a rule not more than 5 to 8 per cent. of the original solids. At this stage the liquid was highly discolored (dark), and the residue had the color of beef muscle. The most intense putrefactive odor made itself known about the fifth day of the decomposition. It was always of the offensive character.

Chemical examinations were usually made after decomposition had been allowed to go on for seven days. In a few instances this process was interrupted on the fourth day, while on two or three occasions it was continued for two weeks or more.

Hydrogen Sulphide.—This was always present in but small amounts at the time of examination. The black deposits in the mercury bottle, however, always indicated considerable evolution of this gas during the first few days of decomposition. Hydrogen sulphide is, no doubt, one of the first substances which are split off from the proteid molecule during putrefaction, as well as in other chemical transformations of albumins. Even the heating[1] of proteids in a neutral or slightly alkaline solution at 80° C. will bring about the elimination of hydrogen sulphide.

[1] Rettger, *Amer. Journ. of Physiol.*, vi, p. 450, 1902.

Mercaptan.—Mercaptan was always found to be present in decompositions in which the above anaerobes had taken an important part. With pure cultures of the anaerobes, the amount of mercury mercaptid precipitate obtained from 200 c.c. of decomposition fluid varied between 0.07 and 0.12 gram. The volume of the precipitates always appeared greater than the actual figures would indicate. Maximum mercaptan production occurred within the first six or seven days, after which the amount gradually diminished. It was about the same for each of the anaerobes tested.

Indol, Skatol and Phenols.—At no time could either skatol or phenol be detected with any certainty among the products of the anaerobes (pure). Neither did the regulation nitric-acid test show any trace of indol. A faint reaction for this substance was obtained, however, with the naphthaquinonemonosulphonate test. We must conclude, therefore, that neither of these anaerobes produces indol, skatol, or phenol (or at best only minute traces) when grown pure in a putrescible substance like the egg-meat mixture. This observation accords with that of Bienstock, which was made on pure cultures of his B. putrificus in blood fibrin.

Skatol-Carbonic Acid.—This substance was likewise found to be absent from the decomposition flasks of different incubation periods. Not the slightest reaction could be obtained.

Aromatic Oxy-Acids.—Strong color reactions were always obtained for aromatic oxy-acids. They were also easily recognized by their characteristic odor. With seven-day cultures a deep cherry-red color was obtained with Millon's reagent in the final 250 c.c. extracts. It required dilutions of one part of extract with 70 to 75 parts of water, to reduce the reaction to the point at which it was just barely perceptible.

Leucin, Tyrosin and Tryptophan.—Leucin, tyrosin and tryptophan were always present in considerable quantities, both in pure cultures of the anaerobes and when the latter were mixed with the colon bacillus or Proteus vulgaris. Albumoses and peptone could be detected only with considerable difficulty; but they were present, nevertheless, as different examinations have shown.

When the obligate anaerobes were cultivated along with other

bacteria, decidedly different results were obtained, and here again my observations support those of Bienstock. The rate and nature of decomposition depend on the particular kinds of organisms with which the anaerobes were mixed. The B. coli communis and B. lactis aerogenes actually checked the rate of decomposition of the egg-meat mixture, and hence caused a decided decrease in the amount of certain products in the given length of time. In some instances, it required fully ten days to reduce the bulk of solids to the same amount as when the pure anaerobe was used for seven-day periods. In fact this antagonism of the colon bacillus to the anaerobe was most noticeable at all times and in every experiment.

On the other hand, the action of Proteus vulgaris was most favorable to the rapid disintegrating action of the anaerobes. With the association of the proteus organism, a decided destruction of the egg albumin could be seen on the second day, a phenomenon which was noticeable only after the second or third day in a pure culture of the anaerobe. In one instance the bulk of solids was completely reduced by the end of the third day, a task which required a full week for the anaerobe alone.

In mixed growths of the anaerobe and B. coli or B. lactis aerogenes, an appreciable amount of indol and minute quantities of skatol and skatol-carbonic acid were produced. In some instances sufficient indol was present in the final distillate to give a distinct color reaction with nitric acid even after 25 or 30 dilutions. The greatest amount of indol was always obtained with B. putrificus and B. coli mixtures.

The presence of staphylococci and streptococci in the culture flasks exerted no noticeable influence on the rate and nature of the putrefaction.

In some instances when the bacillus of malignant œdema and B. coli had been grown together, no indol whatever could be detected, and on two occasions it was found that the colon bacillus had entirely disappeared from the contents of the flask. There is every indication here of an antagonism which is just the reverse of that mentioned earlier in the paper. If this should be so, some light would be thrown on the quantitative relationships of certain of the intestinal bacteria, particularly in cases of pernicious anæmia.

The relation which the tetanus bacillus bears to putrefactive decomposition is decidedly different from that of the above anaerobes. Although a strict anaerobe, it is unable to attack native proteids like blood fibrin or the egg-meat mixture, and therefore plays no part in the initial stages of putrefaction.

INTESTINAL PUTREFACTION.

The colon bacillus has for a long time been associated by pathologists with intestinal disturbances that are of a putrefactive and fermentative nature. Some writers[1] even claim that a derangement of conditions in the intestinal tract is regularly accompanied by an increased number of colon bacilli in the feces.

I have been making a large number of observations on this point in connection with some forty samples of what seemed to be normal feces, as well as at least fifteen specimens of stools from patients suffering with pernicious anæmia. It is very difficult to draw a general conclusion from the results. There seems to be no definite relationship between the number of colon bacilli and the total count of bacterial colonies which grow on lactose-litmus agar plates. While in several cases the "normal" stools contained comparatively few colon bacilli, other specimens yielded culture plates in which this organism predominated to the extent of 75 or 80 per cent. of the total count.

In a few instances the number of colon bacilli in the stools of the anæmia patients far exceeded the average for the "normal" stools, and on one or two occasions they made up over 80 per cent. of the colonies in the agar plates. On the other hand, samples of stools from a severe case of pernicious anæmia which was treated by Dr. Skinner of New Haven, in his private sanatorium, contained but comparatively few of the colon bacilli (0.5–8 per cent. of total count), the large majority of bacteria colonies consisting of streptococci, and most of the remainder of staphylococci. The colon bacillus count was also very small in one other instance (10–15 per cent.); this was likewise a New Haven case. Members of the proteus group were found to be present rarely, and then in small numbers only.

With but a very few exceptions, the predominating organism

[1] Muir and Ritchie, *Manual of Bacteriology* (American Edition), p. 339, 1903.

in both normal and anæmia stools was either the colon bacillus or intestinal streptococcus. Staphylococci which were apparently of the cereus albus group were also commonly present, and on two occasions, B. mucosus capsulatus was found in considerable numbers.

Several examinations of dogs' feces were also made. The feces of three of the dogs always had a high colon bacillus content. In a fourth case, colon bacilli were regularly few, and staphylococci predominated. All of the dogs were fed on the same bread and meat diet, and appeared to be in the same condition generally.

If the colon bacillus does tend to increase in numbers during abnormal conditions of the intestine, one of two things is highly probable. It may itself be directly injurious and aggravate or cause the disturbance; or, its function may be a protective one. The latter view is firmly supported by Bienstock and by the results of the present investigation.

If we assume that intestinal disturbances commonly have their direct origin in excessive putrefactive and fermentative conditions, the colon bacillus, if present in large numbers, would tend strongly to inhibit the putrefaction. In cases, however, where putrefaction has gone unchecked, the colon bacillus is crowded to the wall and the antagonism is exerted in just the opposite direction. Experiments conducted on the egg-meat decompositions largely support these hypotheses.

Numerous investigators have attempted to obtain bacteria which are strictly putrefactive, from the feces of man and some of the lower animals, but, with rare exceptions, without success. Even after the ingestion of garden soil which contained an abundance of putrificus spores, Bienstock was unable to find this organism in his own feces.

I have also made frequent attempts to observe one or another of the known anaerobes of the putrefactive type in both normal stools and those from anæmia patients, but, with two exceptions, I met with failure. In one instance, the putrificus bacillus was easily identified, and in a very recent specimen of stools from an anæmic an organism in every way resembling the bacillus of malignant œdema was present. Both were recognized by their spore form. It is a question, though,

whether these organisms were originally present in the stools or were mere contamination forms. It is an easy matter for bacteria which are so common everywhere to get into specimens of stools, especially when they are soft, no matter how carefully they may have been collected.

The above examinations for anaerobes were made directly on the feces, with the microscope, and after the inoculation of egg-meat mixtures in large test-tubes. In the latter method, thorough decomposition of the native proteids was an indication of the presence of putrefactive anaerobes; nevertheless, microscopic examinations were always made of these cultures.

While putrefactive anaerobes of the above mentioned type could never be identified in stools or cultures made from them, except in the two cases cited, I have succeeded in the large majority of cases in bringing about some decomposition of the egg-meat mixture, which was accompanied always by characteristic putrefactive odors. The reduction in the bulk of the proteids amounted to from 5 to 30 per cent. of the total volume. This reduction was, as a rule, made more apparent if the tubes of egg-meat mixture had been previously heated at 80° C. for ten minutes, as Klein has advised in connection with the detection of his B. sporogenes capsulatus in feces, sewerage, etc. The principle again involved in the heating is that of destroying antagonistic organisms of the colon bacillus type; for it can be shown readily that B. coli communis and B. lactis aerogenes have a strong inhibiting action on putrefactive organisms.

Up to the present time I have observed but little difference in the proteid-decomposing power of stools from healthy persons and those from pernicious anæmia patients. Further study will undoubtedly throw more light on this question.

It is impossible to find an explanation of the putrefactive action exerted on the egg-meat mixture by feces. It may be due to some one or more of the known anaerobes which regularly escape direct observation, or to unknown and unrecognized forms which are commonly present. Numerous microscopic examinations of stools have invariably shown a large number of long, slender bacilli which stained but feebly by the Gram

method They always resisted cultivation in ordinary media, however, and little could be learned about them.

Finally, the possibility has suggested itself that the Bacillus enteritidis sporogenes of Klein [1] may be at least partly responsible for the putrefactive properties of feces, although it is generally regarded as a purely fermentative organism, and not putrefactive. In at least 50 samples of stools that were examined the characteristic Klein reaction [2] for B. sporogenes capsulatus was obtained. On but two occasions it proved negative. This organism is a strict anaerobe, and has very strong fermentative powers. It is regarded by many as being identical with Welch's Bacillus aerogenes capsulatus.

Observations are now under way to determine whether the Klein bacillus has any putrefactive properties when grown in sugar-free proteids, particularly native proteids.

SUMMARY.

Putrefaction is the work of anaerobes, and of all the organisms examined thus far, of strict anaerobes only. The best known of the putrefactive anaerobes are Bacillus putrificus, the bacillus of malignant œdema and the bacillus of symptomatic anthrax. Egg-meat mixture and blood fibrin are readily decomposed by them, yielding the foul-smelling products that are so characteristic of real putrefaction, particularly mercaptan. In pure cultures of these organisms, however, indol, skatol, and phenol are not produced, or only in very minute quantities.

Except in a few rare instances, putrefactive organisms of the above type have not been observed in feces, either of normal persons or of those suffering with pernicious anæmia, even when the stools had a decidedly offensive odor. In the large majority of cases, however, feces have a more or less putrefactive action on the egg-meat mixture, sometimes causing a reduction in the bulk of the proteid of from 25 to 30 per cent. This action is most pronounced, as a rule, after heating the tubes for 10 minutes at 80° C.

[1] Klein, *Centralbl. f. Bakt.*, etc., xviii, 1 Abt., p. 737, 1895.

[2] Klein, *ibid.*, xxii, 1 Abt., p. 113, 1897; and *Report of Local Government Board*, Supplement, p. 210, 1897–98.

The Bacillus enteritidis sporogenes of Klein is regularly present in human feces, and although commonly regarded as being a purely fermentative organism, it may be at least partly responsible for putrefactive changes in the intestine.

The observations recorded in this paper strongly support the view of Bienstock that the Bacillus coli communis and Bacillus lactis aerogenes are not harmful inhabitants of the intestine, as is so often thought, but that their function is a protective one. Their presence in any putrefying medium is a hindrance to the work of the putrefactive bacteria, and therefore serves as a check on their activities.

I take pleasure in acknowledging my indebtedness to Dr. C. A. Herter for his most valuable suggestions and personal help in this work.

ON THE ACTION OF LIPASE.

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(Received for publication, March 10, 1906.)

The study of the fermentation of fat presents in some respects very favorable conditions for investigation. The reaction of the hydrolytic cleavage of a simple ester is a direct monomolecular reaction in one stage, and one that is chemically and physically well understood. Fats and esters may be prepared in a state of high purity. The quantitative estimation of a fat or a fatty acid may be accomplished with accuracy and comparative ease. These conditions fulfill the analytical desiderata. The catalytic accelerations of the hydrolyses of esters comprise some of our best studied examples of catalyses. Under these circumstances the use of a lipase possessing as little as possible of the unfortunate labile attribute of ferments as a class promises exceptional results. The lipases of animal origin are not adapted to this end. They are all quite labile, are difficult of preparation and conservation, and present in actual work many other difficulties that seriously impair the validity of the results In the lipases of vegetable origin we possess, however, ferments most admirably adapted to exact investigations.

The question of the identity of the animal and vegetable ferments is a vain discussion. Both animal and vegetable lipases are able to accelerate the cleavage of all the simple esters of the monatomic alcohols and of glycerin, as well as of many synthetic esters. The statement of Arthus[1] that animal lipase is a monobutyrinase is a verbal quibble. The acceleration of the hydrolysis of these different esters manifested by the ferments varies with the different animal and vegetable lipases, but this furnishes no ground for a segregation. Lipase of animal origin is able to accelerate the hydrolysis of the glycerides of the lower fatty acids; the negative results of some investigators rest upon some error in the experiments.

[1]Arthus, *Journ. d. physiol. et path.*, iv, p. 455, 1902.

The investigations to be here reported were carried out with the lipase of the castor-bean (*Ricinus communis*) first described by Green.[1] This ferment is characterized by an exceptional stability, manifested in a marked resistance to hydrolysis and bacterial decomposition. This quality is so marked that it has permitted of the commercial use of this ferment for the purpose of the manufacture of glycerin from refuse fats. It is prepared with little difficulty in large quantities, and may be conserved with little loss in enzymic properties through long periods of time. Fresh, fully ripe castor-beans are freed of their shells, mashed, and extracted with water-free ether until freed of lipoids. This operation is a tedious process. The residue requires multiple pulverizations between the extractions, but eventually the powder is free of fat and is dry, very light and fluffy, and of a pale yellow color. It is possible also to use the refuse residue of the commercial compression of the castor-oil; too often, however, a high temperature is developed in the process of compression, and the lipase is largely destroyed. This powder keeps indefinitely in the dry state.

The powder must be freed of fat in order that the substrate in the experiment shall not be invalidated in the quantitative sense. One might estimate the fat in the castor-beans, and then allow for it in the tests; but under such circumstances a proper mixing of the fatty powder is not possible, and this mixing is necessary in order that the enzymic strength of the preparation shall be uniform. The ferment is not evenly distributed throughout the different tissues of the bean. It would in any event be very undesirable to have in the system, in addition to the single pure fat that has been selected as the substrate, a mixture of the different fats and lecithins of the castor-bean.

Ferment is lost in this process. This loss affords a very pretty illustration of the principle of partition. Lipase is entirely insoluble in ether; it is, however, quite soluble in ether containing a fatty body. (I use the term soluble in the ordinary sense; in the strict sense the ferment is a suspended colloid.) The different fractions of ether employed in the successive extractions contain quantities of ferment proportional to the fat content.

[1] Green, *Proc. Roy. Soc.*, xlviii, p. 370, 1890.

If a lipase preparation be mixed with water and ether, the ferment will pass into the water to some extent; it will not pass into the ether; the larger portion of the ferment will remain in the third layer. If an ester soluble only in ether be introduced into the system, lipase will pass into the ether. If an ester soluble in both ether and water be introduced into the system, lipase will pass into the ether, and also into the water phase to a greater extent than into simple water. This property is not confined to the lipase of the castor-bean powder, the endotrypsin and invertase exhibit also the same behavior. It is therefore probable that the phenomenon is related to the formation of a ferment-lipoid complex, rather than to the combination of lipase and fat in their capacities of substrate and ferment. The colloidal proteins have physico-chemical relations to the lipoids entirely independent of their fermentative relations.

The lipase acts best in an acid reaction. The optimum concentration is not narrow, rather high concentrations of acetic acid being tolerated without depression. One may safely employ 2.5 per cent. solutions of the triacetin, without fear that the acetic acid evolved will depress the reaction. On the contrary, one may add alkali and so neutralize the acetic acid that the acidity is kept quite low, without disturbing the progression of the reaction.

This castor-bean powder contains several ferments. I have definitely determined lipase, amylase, invertase, maltase, and an endotrypsin to be present. A peroxydase is not present. In addition to these are different proteins—globulin and albumin, nucleo-albumin, and a glycoprotein. It is possible to separate the lipase from most of these other substances. It is, however, not desirable to do so. The presence of the proteins protects the lipase from the action of the endotrypsin. The more purified the lipase, the less stable is it. It is possible to show that with the use of the original powder the lipase retains its full activity in a fermenting system for days; with the use of partially purified powder this cannot be observed.

This powder forms with water a turbid suspension. If filtered through paper, an opaque solution is secured that contains some lipase but much less than the original suspension. The ferment will pass through some infusorial filters to some extent; through

others it will not pass at all. It is quantitatively retained by successive filtrations through paper.

The ferment is very resistant to heat. When dry it may be heated to over 100° C. without injury. Suspended in water it is hydrolyzed on heating; nevertheless, in an acid reaction the destruction is much less rapid than in an alkaline reaction. In the presence of a fat, heating is well borne; this is another illustration of the general fact that ferments are protected by their substrates from the disintegration of hydrolysis.

As substrate I have employed largely the triglyceride of acetic acid, commonly termed triacetin. This ester may be prepared in a state of almost absolute purity. It is best prepared as suggested by Seelig[1]; the diglyceride is first prepared, and then the triacetin obtained from it by prolonged heating with an excess of anhydrous sodium acetate and acetic anhydride. The ester should be purified by distillation at low pressure. This ester possesses many advantages. It is very stable; water-free, it is practically permanent. Watery solutions are hydrolyzed very slowly at ordinary temperature; a 1 per cent. solution will yield scarcely a demonstrable trace of acidity in a month at room temperature. Acids of course accelerate this hydrolysis, but not with marked rapidity. The factor of auto-catalysis is therefore reduced to a minimum, and may be disregarded in ordinary tests of moderate duration. The hydrolysis of the ester is very easily accelerated by lipase. Triacetin is much more easily fermentable with lipase than is ethyl acetate. Ethyl acetate is much more easily split by acids than is triacetin. The esters of the simple monatomic alcohols are indeed quite resistant to the castor lipase, and the results of quantitative tests are irregular and unsatisfactory. The statement of Connstein[2] that the Ricinus lipase acts only on the triglycerides of the higher fatty acids—and by implication not on triacetin—is incomprehensible to me, since I have always found it active. Lipase of animal origin is very active in the acceleration of the hydrolysis of triacetin; it constitutes, in my opinion, the very best routine test for a fat-splitting ferment. The extent of reaction is readily determined by a titration.

[1] Seelig, *Ber. d. deutsch. chem. Gesellsch.*, xxiv, p. 3466, 1891.
[2] Connstein, *Ergebnisse d. Physiol.*, iii, 1, p. 231, 1904.

The reversed reaction, the formation of triacetin from glycerin and acetic acid, though demonstrable, is a reaction of very low velocity, so low that the factor may be disregarded in ordinary tests. The reaction of cleavage is, however, never complete; there is a definite point of equilibrium in the reaction, as there seems to be in all systems of the order of ester + water $\leftrightarrows$ fatty acid + alcohol. The position of the station of equilibrium may be determined by carrying out an acid hydrolysis until the titration becomes stationary. This I have done for 0.5, 1.0, and 2.0 per cent. solutions of triacetin. The hydrolysis was accomplished by means of normal acid, adding an equal volume of a normal sulphuric acid to the solution of triacetin and allowing the system to lie for several months until the equilibrium was reached. It is not possible to employ a high temperature, since the station of equilibrium may be shifted by alteration in temperature. The 0.5 per cent. solution when in equilibrium has the composition, ester 12 : products 88; the 1 per cent. solution has the composition, ester 18 : products 82; and the 2 per cent. solution has the composition, ester 22 : products 78. When these are compared with the equilibria observed in similar systems hydrolyzed with lipase, it is noted that approximately the same values are observed. Thus the completed fermentation of the 0.5 per cent. solution has the composition, ester 14 : products 86; the 1 per cent. solution has the composition, ester 21 : products 79; while the 2 per cent. solution has the composition, ester 30 : products 70. From these data we may reason that the lipase does not shift the station of equilibrium, *i. e.* unlike most ferments, it acts as a pure catalysor.

There have been few studies on the quantitative relations in the action of lipase on a soluble substrate. Kastle and Loewenhart[1] and later Kastle, Johnston, and Elove,[2] using animal lipase, found that the transformation was not closely proportional to the substrate concentration; there was a lagging in the constants. This lagging they attributed to the influence of the fatty acid. The ferment was found to be quite resistant to inactivation while in the digesting system. The investigations

[1] Kastle and Loewenhart, *Amer. Chem. Journ.*, xxiv, p. 491, 1900.
[2] Kastle, Johnston, and Elove, *ibid.*, xxxi, p. 521, 1904.

of Vollhard,[1] Stade,[2] Fromme,[3] Engel,[4] Connstein, Hoyer, and Wartenberg,[5] Nicloux,[6] Henri and Nicloux,[7] Zellner,[8] and Kanitz[9] are not comparable directly with the experiments on digestion of a soluble substrate, since they worked with bi-colloidal systems.

The lipase of the castor-bean yields exceptionally good quantitative results in experiments on the reaction kinetics of the process. I have usually added toluol to the tests. One could with careful technique dispense with any antiseptic, since the powder and the triacetin are both sterile. The methylbenzol, however, does not in the least disturb or modify the action of the ferment and the technique is simpler when it is employed. The following table contains the measurements on three sets of tests carried out at a uniform temperature of 18° C., with solutions of varying strength. The ferment concentration was 1 gram of the powder to the volume of 100 c.c. For each measurement there was a separate test. This was made necessary by the fact that it is not possible to obtain with a pipette from a large mass ot the mixture a portion that may be trusted to be representative of the whole. With the use of multiple tests one avoids this difficulty, but one has then the results exposed to the variations in the weighing of the substrates and ferment for the several tests, errors that are of no little moment, since the powder is difficult to weigh on account of the magnetic property that causes it to fly apart when touched. The measurement was made by a titration of the filtrate. The results are calculated according to the monomolecular order of the reaction by the equation, $c = \frac{1}{t} \log \frac{A}{A-x}$, derived by integration from the equation $-\frac{dx}{dt} = c\,(A-x)$. The constants are $\times 10^{-4}$.

1 Vollhard, *Zeitschr. f. klin. Med.*, xlii, p. 414, 1901; xliii, p. 397, 1901.
2 Stade, *Beitr. z. chem. Physiol. u. Path.*, iii, p. 291, 1903.
3 Fromme, *ibid.*, vii., p. 51, 1905.
4 Engel, *ibid.*, vii, p. 77, 1905.
5 Connstein, Hoyer, and Wartenberg, *Ber. d. deutsch. chem. Gesellsch.*, xxxv, p. 3988, 1902.
6 Nicloux, *Compt. rend. de soc. biol.*, lvi, p. 840, 1904.
7 Henri and Nicloux, *ibid.*, lvii, p. 175, 1904.
8 Zellner, *Monatsh. f. Chem.*, xxvi, p. 727, 1905.
9 Kanitz, *Zeitschr. f. physiol. Chem.*, xlvi, 482, 1905.

t (hrs.)		4	8	16	24	28	32	40	48
0.5 %	x	0.096	0.162	0.287	0.418	0.489	0.477	0.623	0.652
	$\overline{A}$ C	109	96	92	98	104	88	106	97
1 %	x	0.083	0.174	0.338	0.418	0.488	0.542	0.609	0.655
	$\overline{A}$ C	94	104	112	98	104	106	102	96
2 %	x	0.098	0.174	0.323	0.431	0.502	0.485	0.595	0.636
	$\overline{A}$ C	112	104	106	102	108	90	98	91

Despite the notable variations in the constants, they are, when the conditions of the experiment are taken into consideration, in good agreement. Most important is the agreement in the constants of the different series with varying substrate concentrations. This agreement warrants the statement that in the fermentation of triacetin by lipase the transformation in the unit of time is proportional to the mass of the substrate.

I have not been able to obtain a similar result with the corresponding ester of a simple alcohol. As before mentioned, the esters of the simple alcohols do not behave with lipase as do the esters of glycerin. Thus, for two different series with ethyl acetate the following constants were obtained. The constants are $\times 10^{-6}$.

1 per cent.—	203	264	246	221	186	234	164	238
2 per cent.—	390	413	322	253	433	410	298	436

The agreement between the constants in each series is not good,—not so good as obtained by Kastle, Johnson, and Elove.[1] What makes them, however, of very little value is the fact that the constants for the two series are not at all in agreement. This is the difficulty that has been so often met with in fermentation tests by various authors. No one has yet obtained an agreement in the constants for different initial substrate concentrations in the fermentation of protein, starch, glucose, or of glucosides; and such agreement has been obtained in the

[1] *Loc. cit.*

inversion of saccharose, maltose, and lactose by Henri[1] and Armstrong[2] only with the use of the special equation of Henri.[3] The agreement of the constants in the fermentation of triacetin and the lack of agreement in the fermentation of ethyl acetate indicate that we must not look entirely to the ferment for the cause of deviations in the quantitative progression of a fermentation. I have found the same fact to hold for the lipase of the pancreatic juice; it accelerates the hydrolysis of triacetin much more actively and regularly than that of ethyl acetate.

One theoretical objection might be made to the direct application of the law of the monomolecular reaction to the reaction of the hydrolysis of a triglyceride; namely, that the reaction occurs in three stages. That the three molecules of fatty acid are successively split off is theoretically probable, since it is known that in the synthesis of the triglycerides they are added successively. The experimental demonstration of the existence of a monoglyceride or a diglyceride in the system at any moment of the reaction has, however, never been conclusively accomplished, and if the reaction occurs in stages, these are apparently very transient. The experimental results indicate that if the reaction is one in multiple stages, the relative progression is so regular that the experimental velocity corresponds to that of a reaction of the first order in a single stage.

The influence of increase of temperature on the velocity of the lipase fermentation of triacetin was tested by the use of the empirical equation of van't Hoff:

$$\frac{\text{Velocity at } Tn + 10}{\text{Velocity at } Tn} = 2+.$$

Employing temperatures 18° and 28° C., the average of several series was 2.6. The figures vary somewhat with different preparations and concentrations, nevertheless they correspond directly with what would be expected with a chemical reaction in a homogeneous system.

My experiments on the hydrolysis of the higher glycerides have been limited to triolein. Against the employment of natural

1 Henri, *Lois générales des diastases*, 1903, p. 93.
2 Armstrong, *Proc. Roy. Soc.*, lxxiii, pp. 500, 516, 1904.
3 *Loc. cit.*, p. 85.

fats composed of the triglycerides of oleic, palmitic, and stearic acids, theoretical objections based upon the impossibility of controlling the substrate concentration may be urged. When we speak of a fluid natural fat, we mean a fat in which at the temperature present the triolein is able to hold the tripalmitin and tristearin in solution. The triolein is the solvent for the other two esters. The so-called melting-point of a natural fat is not a physical melting-point at all; it represents the temperature at which the mass of triolein in the fat under observation is able to hold in solution the tristearin and tripalmitin, it is a phenomenon of solution, of saturation, and does not correspond to the transfer of a substance from the solid to the fluid state. Analagous relations hold for oleic acid. It is a solvent for triolein, and also for tripalmitin and tristearin. Furthermore, triolein dissolved in a watery solution of oleic acid is a better solvent for the other two esters than is a solution of triolein in water. There is some evidence that in the fermentation of a mixed fat the tristearin is first and most easily attacked. In any event, it is obvious that with the facts as stated, we are confronted with the condition that in the system under observation one of the products of the reaction increases the solubility of the substrate; and with the substrate and the products both participating in the rôle of the solvent, the substrate concentration cannot be controlled. This control of the substrate concentration is however the *conditio sine qua non* of the application of the equation for the reaction, and thus the impossibility of securing accurate results in the digestion experiment with mixed fats is apparent. Vollhard,[1] Stade,[2] Fromme,[3] and Engle,[4] worked with gastric lipase, and used the yolk of egg for substrate. In the experiments of Vollhard, Stade, and Fromme the results were irregular. In the results of Engel the constants conformed in a rough way with the equation $C = \frac{x}{\sqrt{t}}$, though the constants fell progressively. Connstein, Hoyer, and Wartenberg[5] studied the action of lipase of the castor-bean upon different natural

[1] *Loc. cit.*
[2] *Loc. cit.*
[3] *Loc. cit.*
[4] *Loc. cit.*
[5] *Loc. cit.*

fats. They did not carry out any controlled, quantitative tests, but a recalculation of their results indicates that the reaction was not a function of time, nor proportional to the substrate concentration. They determined that the higher the fatty acid in the scale, the more easily was its triglyceride fermentable. Nicloux,[1] working with the Ricinus lipase and olive-oil, found that with high concentrations of the ferment, the transformation was roughly proportional to the mass of substrate. Henri and Nicloux[2] found that with low concentrations of the ferment the transformation was constant in the unit of time. Zellner[3] studied the lipase of *Amanita muscaria.* Working with olive-oil he found the transformation constant in the unit of time; with tallow, he obtained an irregular result. Kanitz[4] has most recently studied pancreatic lipase, and he seems to have been able to secure much more active preparations than has been the fortune of most investigators. He found the transformation of olive-oil was not a function of time, but proportional to the square root of the time. In none of the above investigations was mechanical shaking employed, though the theory demands it.

Even with the employment of a single ester of the higher fatty acids, such as triolein, it is apparent that anomalous conditions are in hand. One is working with a bi-colloidal system. The ferment is in colloidal suspension, the fat is in colloidal suspension if a proper emulsion be secured. The reaction may be probably assumed to occur only at the boundary of contact of the ferment phase, though this is not certain. Two streams of diffusion are therefore obviously necessary instead of the single stream of the substrate to, and the products from, the film of reaction, as in the case of the hydrolysis of a soluble fat by lipase. Working with low concentrations of lipase corresponding to those that were employed in the other tests and using purified triolein as a substrate, I have obtained the following results. Regular and systematic mechanical shaking is essential in these experiments.

1 *Loc. cit.*
2 *Loc. cit.*
3 *Loc. cit.*
4 *Loc. cit.*

Two per cent. suspension of triolein, ferment powder 1 per cent., temperature 18° C. The results are in terms of cubic centimeters of decinormal acid; t is days.

t	1	2	3	4	5	7	9	11	14	18
x	3	5	7.5	9	11	16	21.6	27.2	33.1	38.1

These give the following increases in acidity for each day: 3—2.5—1.5—2—2.5—2.5—2.8—2.8—2.8—2.2—2.2—2.2—2.2—2.7—2.7. Though it must be stated that there is quite a possibility of error in the estimation of the acidity—an extraction with ether, followed by the titration in alcohol of the ethereal extract—it is apparent that the transformation is a linear function of time, $\frac{x}{t} = C$. Henri and Nicloux[1] obtained similar results. They found that there was for the castor-bean lipase an optimum concentration of acidity; when at this concentration of acidity different concentrations of substrate were split with the lipase, the transformations in all were approximately identical in time. Zellner[2] obtained a similar result with the mushroom lipase.

This experimental result—that the velocity is a function of time—might be theoretically interpreted from three points of view: (*a*) the experimental velocity represents simply a reaction velocity in a homogeneous system at a maintained saturation of the substrate; (*b*) the experimental velocity represents a superimposition of reaction and diffusion velocities; (*c*) the experimental velocity represents a diffusion velocity alone.

(*a*) The experimental result that the transformation was constant in time might be held to be in accordance with the idea that the reaction was essentially a monomolecular reaction in a homogeneous system. If in such a system the substrate be maintained constant and the products of the reaction removed, the transformation ought to be a direct function of time. In the present instance it may be assumed that as fast as a molecule of fat was hydrolyzed and the fatty acid passed out of solution, a corresponding molecule of fat would pass into solution from the suspended excess; thus the substrate concentration would remain constant, and the products would not accumulate to depress the reaction. These being the relations, the trans-

[1] *Loc. cit.*
[2] *Loc. cit.*

formation would follow the equation: *Const. Concentr.* $= \frac{x}{t}$. This proposition obviously regards the suspended fat as inactive in the sense of the law of mass action, the soluble fat only as active. It is, however, an error to suppose that the saturation would be uniform through the course of such an experiment. The solubility of the fat is influenced by the presence of the fatty acid. Supersaturation would be certain to occur at the line of contact of the two phases, and the degree of supersaturation would be in a sense proportional to the dimensions of the boundary of contact of the substance to be dissolved, at least if proper stirring be maintained. It is apparent that in this hypothesis the assumption is tacitly made that of the two processes, that of solution of the substrate and of the reaction of the substrate with the ferment, the velocities are uniform and constant. Now the velocity of the solution of the substrate is certainly a diffusion velocity, and the attempt to regard the measured velocity of the fermentation purely as a reaction velocity in a system at constant saturation is not permissible, unless one makes the assumption that the diffusion occurs with practically infinite rapidity as contrasted with the reaction. For this last assumption there is no evidence, quite to the contrary.

(*b*). If the experimental velocity be regarded as a superimposition of the reaction and diffusion velocities, it follows that the concordance of the experimental result with the equation $C = \frac{x}{t}$ was merely a coincidence. The diffusion and reaction velocities have different temperature coefficients; the coefficient of solubility would vary greatly with temperature; the supersaturation cannot be regarded as constant on account of the progressively lessening surface of the suspended fat and because of the influence of the fatty acid on the solubility of the fat. If, therefore, at any particular temperature the experimental velocity follows the equation $C = \frac{x}{t}$, that would be a coincidence, and could not hold for different temperatures. Now the results in experiments at different temperatures, as I have learned, express the same mathematical relation. The experimental velocity in the unit of time is simply somewhat faster at higher temperature. That the experimental temperature coefficient is low, as will be noted later, speaks directly

against the idea that the experimental velocity represents a superimposition of a diffusion and a reaction velocity.

(*c*). If the experimental velocity is to be interpreted as representing a diffusion velocity solely, it is implied that the time consumed in the chemical reaction of hydrolysis is practically nil as compared with the time consumed in the processes of diffusion. As above elucidated, the experimental result is scarcely to be explained on the basis of a reaction velocity in a reaction of the first order. Even were we to assume, as has been customary, that under the circumstances of a reaction in a heterogeneous system the velocity of reaction is proportional to the active mass of the reacting bodies and to the dimensions of the surface of contact, the present result is anomalous. That the active substrate concentration was constant during this experiment cannot be granted, though the triolein was in saturated solution after a certain amount of oleic acid was produced. The dimensions of the surface of contact cannot be believed to have remained constant, unless we assume that the relation between the water and the ferment was independent of the triolein and the oleic acid. As a matter of theory we are driven to the conclusion that the phase relations in the system represent an equilibrium between the triolein, oleic acid, glycerin, ferment, and water. The system water-triolein is a two-phase system in the strict sense of the word. The triolein is almost insoluble in water. With the advent of the oleic acid, much more of the triolein will be taken into solution. We will then have a water-rich, triolein-oleic-acid-poor phase, and a water-poor, triolein-oleic-acid-rich phase. When lipase is mixed with water, we have a two-phase system, a water-rich, ferment-poor phase, and a water-poor, ferment-rich phase. At the boundary of contact of the phases in each instance there is a zone of hypersaturation, a phenomenon of adsorption. When these two systems of phases are combined, as occurs in the experiment, an equilibrium must obviously occur between these relations (*cf.* Henri[1]). As the digestion proceeds, *i. e.*, as the fat is split and glycerin passes into the water and oleic acid passes out of solution in each minute, these phase relations must be shifted.

[1] Henri, *Zeitschr. f. physik. Chem.*, li, p. 19, 1905.

In the beginning of the experiment, the colloidal phase contains much triolein; at the close of the experiment, principally oleic acid. To these alterations of the substrate, the partition of the ferment phase must adjust itself.

Under these circumstances the experimental result, that the transformation is a linear function of time, is best explained on the Nernst[1] hypothesis that the experimental velocity is not a reaction velocity in the chemical sense, but a diffusion velocity in the physical sense. The theory of Nernst is based upon the proposition that chemical reactions occurring at the boundary of contact of two phases do so with practically infinite velocity. Time would therefore be consumed only in the diffusions to and from the film of contact of the two phases where the chemical reaction occurs. This excludes the application of the van't Hoff *theory of the order of a reaction to reactions in a hetero*geneous system. This hypothesis is an expansion of the theory of Noyes and Whitney[2] of the solution velocity of a solid in a fluid. If the reaction at the film of contact of the two phases occurs with great rapidity and the homogeneity of the general medium be secured by adequate stirring, the time consumed in the experiment will be utilized entirely by the diffusion of the substrate to and of the products from the zone of reaction; *i. e.*, the experimental velocity is a diffusion velocity. Under the conditions of concentration present in the triolein experiment, the transformation in the unit of time ought to be constant. If now the experimental velocity be a diffusion velocity instead of a reaction velocity, this fact ought to be directly demonstrable by experiment. As is well known, the temperature coefficients of chemical reactions and of diffusion velocities exhibit very different orders of magnitude. For ordinary chemical reactions the empirical rule of van't Hoff, that the velocity of a reaction is at least doubled for every ten degrees increase in temperature, will suffice. A similar increase in temperature will ordinarily increase a diffusion velocity but slightly—less than one-sixth. I have repeated the above experiment and have determined that an increase of ten degrees accelerates the experimental velocity only 20 per cent. A similar result was obtained by

1 Nernst, *Zeitschr. f. physik. Chem.*, xlvii, p. 52, 1904.

2 Noyes and Whitney, *ibid.*, xxiii, p. 689, 1897.

Kanitz.[1] Senter[2] has found the same fact to hold in the reaction of hydrogen peroxide-hamase and has drawn the corresponding conclusion that the experimental velocity is a diffusion velocity.

Kastle and Loewenhart[3] determined the relation of ferment mass to degree of acceleration to be one of direct proportionality. The same relation holds true for the Ricinus lipase as illustrated by the following:

Substrate (triacetin) constant. In (a), ferment 2 per cent.; in (b), ferment 1 per cent. The times given are those necessary for the proportional transformation of the stated units of acid.

Units	10	20	30
(a) *t*	23	51	104
(b) *t*	47	97	216

Vollhard[4] and Stade[5] and Engel,[6] working with yolk of egg, obtained figures that corresponded roughly to the so-called rule of Schütz, which is also believed by the Pawlow school to hold true for animal lipase; Fromme could not confirm it. Kastle and Loewenhart,[7] who obtained results contrary to these investigators, worked like them with animal lipase. From the considerations elucidated above it is certain that digestion experiments in a bi-colloidal system are not adapted to the determination of this question. The newer studies on ferment action have thrown doubt upon the so-called rule of Schütz. For many ferments it is now known that the acceleration is proportional to the mass of ferment. Quite recently Sawjalow[8] of the Pawlow school has admitted that the Schütz rule holds for peptic digestion only when the tubes of Mett are employed. The Mett method, however (the measurement of the linear digestion

[1] *Loc cit.*
[2] Senter, *Zeitschr. f. physik. Chem.*, lii, p. 737, 1905.
[3] *Loc cit.*
[4] *Loc. cit.*
[5] *Loc. cit.*
[6] *Loc. cit.*
[7] *Loc. cit.*
[8] Sawjalow, *Zeitschr. f. physiol. Chem.*, xlvi, 6

of a cylinder of coagulated substrate in the unit of time), is theoretically not adapted to the physico-chemical problem in hand.

The ability of lipase to accelerate the reversed reaction, the formation of the ester from the fatty acid and the alcohol, first determined for animal lipase by Kastle and Loewenhart[1] has been confirmed for the pancreatic lipase by Pottevin[2] and for the Ricinus lipase by myself[3]. Pottevin mixed the dry alcohol and the fatty acids with the lipase (pancreatic powder) and reported positive results. I was unable in a previous experiment to confirm these results. More recently, however, I have also observed the synthesis of triolein from dry glycerin and oleic acid through the agency of pancreatic powder, but I obtained again negative results with palmitic and stearic acids. *Unquestionably the fault lies in my experiments, not in those* of Pottevin. Dry lipase of the castor-bean will synthesize all the higher fats from the dry glycerin and fatty acids. The idea of Pottevin however—that lipase acts as a hydrolyzing agent in the presence of water and as a synthetic agent in the absence of water—is not supported by experimental evidence and is based upon a theoretical misconception. All syntheses of fat in nature occur in the presence of water; and the theory of chemical equilibrium cannot be adduced in support of the Pottevin suggestion, since this amounts practically to the statement that the lipase accelerates the reaction of synthesis in the absence of water when it is a bimolecular reaction and accelerates the cleavage in the presence of water when it is a monomolecular reaction. That all reversions at the temperatures employed are very much slower than the reactions of cleavage is true; the theory of reversed reaction does not, however, postulate anything with regard to the velocity. In special instances good theoretical and experimental reasons have been adduced to explain the slow velocity of the reversed reactions; no doubt such exist for all.

Pertinent in this connection are a few general observations on the bearing of the phase relations on chemical reactions occurring

[1] *Loc. cit.*
[2] Pottevin, *Compt. rend. de l'Acad. des sci.*, cxxxviii, p. 378, 1904.
[3] Taylor, *Univ. of Cal. Pub., Path.*, 1904, i, p. 33.

in organized bodies. It seems certain that the theory of Nernst—that reactions occurring at the boundary of contact of two phases do so at practically infinite velocity—is destined to have a deep influence on our conceptions of biological processes. The circulating fluids of the body are two-phase systems in the sense that hydrosols are two-phase systems. The cellular elements of the body are two-phase systems in the sense that hydrogels are two-phase systems. The protein-lipoidal complexes are in cells arranged in colloidal and watery phases, described by Quincke as a meshwork. Of these there are innumerable units in the body, presenting boundaries of contact of almost unimaginable magnitude. We may assume that the chemical reactions of the body occur in large part not in the circulating fluids but in the cells. And in the cells we may assume that they occur at the film of contact of the two phases. On the assumption that the ferments are formed in the colloidal phase of the cells and are spread over the water-poor phase as a thin layer to which the substrates of the different reactions are transported by the circulating streams, we have here realized in an ideal sense the exact relations postulated in the theory of Nernst. A rapid reaction at the boundary of contact would represent an enormous velocity of transformation. When we contemplate the relations of the reactions in the nutrition of the body, we perceive the direct application of these considerations. One of the most striking facts in the study of fermentations is the discrepancy between the velocities of reaction *in vitro* and *in vivo*. This is not so marked for the reactions of decomposition, where we are often able to secure comparative results. But it is very marked for the reversions, the syntheses. Let us assume that the fatty acids and glycerin that have been separated in the digestion of a fat within the intestine are absorbed as such and then in the intestinal mucosa reunited through the agency of lipase. The reversed reaction *in vitro* is very slow; it is very rapid *in vivo*. It is possible in the intestine to synthesize within an hour an amount of fat that would require months in glass. Is it not probable that this great velocity of synthesis in the intestinal mucosa is due to the fact that the reaction is occurring at the film of contact of two phases? The lipase may be assumed to lie as a thin layer over the surface of the meshes of the col-

loidal phase of the cells and over this surface are poured the fluids of resorption containing the fatty acids and glycerin. Thus we would have the ideal conditions postulated by the hypothesis. The possible presence and influence of co-ferments or zymo-excitors cannot be denied and their influence may be super-imposed. But it seems logical and proper, in view of the experimental demonstration of the Nernst hypothesis on inorganic material and of the occurrence in tissues of the physical conditions postulated by the hypothesis, to apply these considerations to the problem of the velocity of chemical transformations occurring within the body apparently under the influence of ferments. It is not necessary to assume, if we apply these considerations to an organism, that in view of the circulatory activity the conception of reaction velocity would be wholly supplanted by that of diffusion velocity; relations of distribution and equilibrium would have to be taken into account, and as the consideration concerns only the velocity and not the qualitative nature of the reaction, the validity of the chemical point of view is not disturbed. But from the point of view of a dynamic physico-chemical conception of biological processes, the importance of this hypothesis must be patent.

III. RESEARCHES ON PYRIMIDINS: 5-ETHYLCYTOSIN.

(Eighteenth Paper.)

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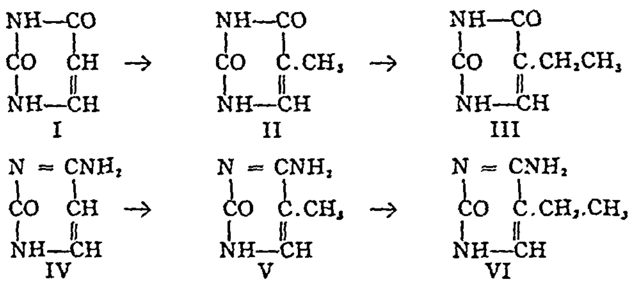
(Received for publication, May 13, 1906.)

Methods of synthesizing uracil, I, thymin, II, cytosin, IV, and 5-methylcytosin, V, have been described in previous papers from this laboratory.[1]

Thymin, II, and 5-methylcytosin, V, are the simplest mono-5-alkyl derivatives of uracil, I, and cytosin, IV, respectively. They may be considered as the second members of two homologous series of which uracil, I, and cytosin, IV, are the primary substances.



The work described in this paper was undertaken with the object of preparing 5-ethyluracil, III, and 5-ethylcytosin, VI, the third homologues of these two series.

We find that ethylformate condenses with normal ethylbutyrate in the presence of sodium ethylate, or metallic sodium, to form the sodium salt of ethyl formylbutyrate, VII:

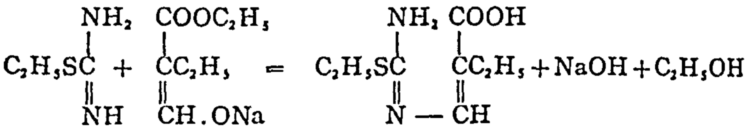
$$CH_3.CH_2\,CH_2COOC_2H_5 + HCOOC_2H_5 + Na = CH_3.CH_2.C{:}(CHONa)COOC_2H_5 + C_2H_5OH$$

VII

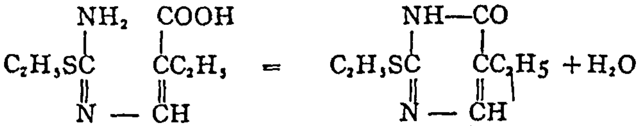
When this sodium salt, VII, was dissolved in water with the

[1] Wheeler and Merriam, *Amer. Chem. Jour.*, xxix, p. 478, 1903; Wheeler and Johnson, *ibid.*, xxix, p. 492, 1903; *ibid.*, xxxi, p. 591, 1904.

calculated quantity of pseudoethylthiourea they condensed to form 2-ethylmercapto-5-ethyl-6-oxypyrimidin, IX. This condensation involves the intermediate formation of α-ethyl-β-pseudoethylthioureaacrylic acid, VIII. We have succeeded in isolating this compound.

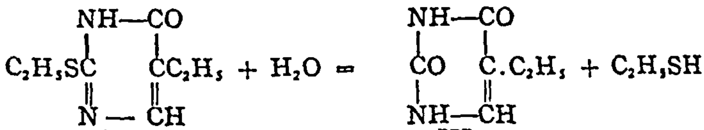


VIII



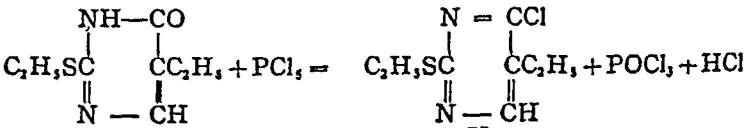
IX

Practically a quantitative yield of 5-ethyluracil, III, was obtained when this mercaptopyrimidin, IX, was boiled with hydrobromic acid:



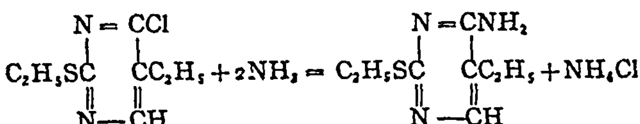
IX III

When 2-ethylmercapto-5-ethyl-6-oxypyrimidin, IX, was heated on the steam-bath with phosphorus pentachloride it was converted into 2-ethylmercapto-5-ethyl-6-chlorpyrimidin, X:



X

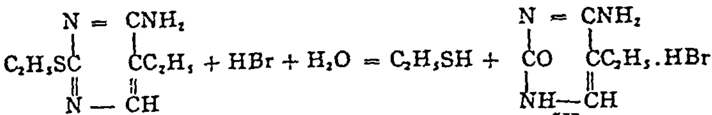
This mercaptochlorpyrimidin, X, then gave a quantitative yield of 2-ethylmercapto-5-ethyl-6-aminopyrimidin, XI, when heated with alcoholic ammonia:



XI

Finally this 2-ethylmercapto-5-ethyl-6-aminopyrimidin, XI,

was converted into the hydrobromide of 5-ethylcytosin, VI, by boiling with hydrobromic acid.



VI

5-Ethylcytosin, VI, separates from water without water of crystallization. This property is of especial interest. Cytosin, IV, and 5-methylcytosin, V, the preceding members of the same series, crystallize with one molecule and a half molecule of water of crystallization respectively.

$C_4H_5ON_3.H_2O$,	$C_5H_7ON_3.\frac{1}{2}H_2O$,	$C_6H_9ON_3$.
(Cytosin)	(5-Methylcytosin)	(5-Ethylcytosin)

5-Ethylcytosin is more soluble in water than cytosin, and less soluble than 5-methylcytosin. It forms normal salts (1:1) with hydrochloric, hydrobromic, and nitric acids.

One of the most characteristic properties of 5-methylcytosin was its tendency to form basic salts[1] with hydrobromic and hydrochloric acids. We now find that 5-ethylcytosin likewise possesses this interesting property. When ammonia was added to an aqueous solution of the hydrobromide we obtained a mixture of two hydrous, basic salts. Our analytical determinations indicated that we were dealing with a 2:1, XII, and a 3:1 hydrobromide, XIII. On account of the small amount of 5-ethylcytosin at our disposal we were unable to make as thorough a study of these basic salts as we desired.

$(C_6H_9ON_3)_2.HBr.H_2O$,	$(C_6H_9ON_3)_3.HBr.H_2O$.
XII	XIII

5-Ethylcytosin is precipitated by phosphotungstic acid. The amino radical is more firmly linked in this base than in cytosin and 5-methylcytosin. While the latter two bases are converted into uracil and thymin when heated with 20 per cent. sulphuric acid, 5-ethylcytosin was recovered unaltered when treated under the same conditions.

[1] Wheeler and Johnson, *loc. cit.*

TABLE OF THE SALTS OF CYTOSIN, 5-METHYLCYTOSIN AND 5-ETHYLCYTOSIN WITH THE COMMON INORGANIC ACIDS: ALSO SOLUBILITY OF THESE BASES IN WATER AT 25° C. AND WATER OF CRYSTALLIZATION.

Base	HCl Salts	HBr Salts	HNO_3 Salts	H_2SO_4 Salts	Water of Crystallization	Solubility in Water at 25° C
N = C.NH$_2$ CO CH NH—CH (Cytosin)	$(C_4H_5ON_3).2HCl$*		$(C_4H_5ON_3).HNO_3$‡	$(C_4H_5ON_3).H_2SO_4$‡ $(C_4H_5ON_3)_2.H_2SO_4$** $(C_4H_5ON_3)_4.H_2SO_4$‡ $(C_4H_5ON_3)_4.H_2SO_4.2H_2O$††	$1H_2O$	100 parts of water dissolve 0.8 parts of base.
N = C.NH$_2$ CO C.CH$_3$ NH—CH (5-Methylcytosin)	$(C_5H_7ON_3).HCl$* $(C_5H_7ON_3).HCl.2H_2O$* $(C_5H_7ON_3)_2.HCl.H_2O$* $(C_5H_7ON_3)_5.2HCl.3H_2O$*	$(C_5H_7ON_3)_2.HBr.H_2O$*			$\frac{1}{2}H_2O$	100 parts of water dissolve 4.5 parts of base.
N = C.NH$_2$ CO C.C$_2$H$_5$ NH—CH (5-Ethylcytosin)	$(C_6H_9ON_3).HCl$†	$(C_6H_9ON_3).HBr$† $(C_6H_9ON_3)_2.HBr.H_2O$† $(C_6H_9ON_3)_3.HBr.H_2O$†	$(C_6H_9ON_3).HNO_3$†		No water	100 parts of water dissolve 1.33 parts of base.

*Wheeler and Johnson, *Amer. Chem. Jour.*, xxxi, p. 598.
†Johnson and Menge, this paper.
‡Kossel and Steudel, *Zeitschr. f. physiol. Chem.*, xxxviii, p. 52.
**Levene, *ibid.*, xxxvii, p. 405.
††Levene, *ibid.*, xxxix, pp. 7, 135, 481.

EXPERIMENTAL PART.

Sodium Salt of Ethyl Formylbutyrate,
$C_2H_5.C:(CHONa)COOC_2H_5$

This salt can be prepared very easily by proceeding in the following manner: A known weight of alcohol-free sodium ethylate was suspended in dry ether. A mixture of the calculated quantities of ethylformate and ethylbutyrate was then added to the ether and the condensation allowed to proceed at ordinary temperature. Sufficient heat was evolved to cause the ether to boil. The mixture was allowed to stand for four days when the excess of ether was removed by sucking a current of dry air through the solution. We obtained a thick oil which immediately solidified to a solid cake when treated with a little water. No further purification was attempted. This salt was dissolved in cold water and the solution washed with ether to remove any unaltered esters. This solution was then used for the condensations described in this paper. In our calculations we have assumed that the sodium salt is formed in practically theoretical amount.

2-Ethylmercapto-5-ethyl-6-oxypyrimidin,
$$\begin{array}{ccc} & NH - & CO \\ & | & | \\ C_2H_5S & C & CC_2H_5 \\ & \| & \| \\ & N - & CH \end{array}$$

One hundred and fifty grams of normal ethylbutyrate were condensed with ethylformate in presence of sodium ethylate as described in the preceding experiment. The resulting sodium salt of ethyl formylbutyrate was then dissolved in water, and treated with an aqueous solution of 0.5 molecular proportion of pseudoethylthiourea. This was prepared by dissolving 119 grams of the hydrobromide in ice water and neutralizing the hydrobromic acid with 36 grams of potassium hydroxide. The alkaline solution was allowed to stand over night at ordinary temperature and then heated on the steam-bath for about two hours. The solution was then filtered and acidified with acetic acid. The mercaptopyrimidin separated at once as a flocculent precipitate. After drying over sulphuric acid the crude material weighed 40 grams, corresponding to 38 per cent. of the theoretical,

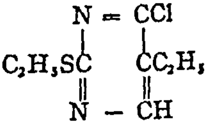
calculating from the weight of pseudourea used. It was extremely soluble in alcohol and benzene, and moderately soluble in ether. It was difficultly soluble in water. It deposited from hot water or dilute alcohol in well developed prisms that melted at 119–120° C. to a clear oil. Analysis (Kjeldahl):

Calculated for $C_8H_{12}ON_2S$:	Found:
N = 15.17 per cent.	15.12 per cent.

α-Ethyl-β-pseudoethylthioureaacrylic acid,
$H_2N(C_2H_5S):N.CH:C(C_2H_5).COOH$.

This was obtained as an intermediate product in the preceding condensation. After filtering from the 2-ethylmercapto-5-ethyl-6-oxypyrimidin the acetic acid filtrate was concentrated on the steam-bath and then allowed to stand at ordinary temperature for several hours. A heavy, flocculent precipitate separated on cooling. The substance was very soluble in alcohol. When heated above its decomposition point it was converted into the mercaptopyrimidin above. It deposited from hot alcohol in plates that melted at 148°–149° C., with effervescence, to a clear oil. When this oil was allowed to cool below 100° C. it solidified. Upon heating again in the capillary tube it melted at 117°–118° C. to a clear oil. Analysis (Kjeldahl):

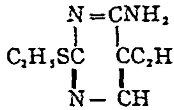
Calculated for $C_8H_{14}O_2N_2S$:	Found:
N = 13.86 per cent.	13.70 per cent.

2-Ethylmercapto-5-ethyl-6-chlorpyrimidin, 

Twenty-five grams of 2-ethylmercapto-5-ethyl-6-oxypyrimidin and a slight excess over one molecular proportion of phosphorus pentachloride were mixed in a flask and gently heated on the steam-bath. A violent reaction took place with evolution of hydrochloric acid gas. We obtained a dark colored liquid that was heated for several minutes at 100° C. to complete the reaction. The liquid was then poured upon crushed ice to decompose the phosphorus halides. The chlorpyrimidin was extracted with ether, dried over calcium chloride, and purified by distillation under diminished pressure. It boiled at 160°–163° C.

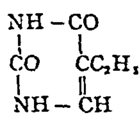
under 24 mm. pressure. It would not solidify in a freezing mixture. The yield of pure distilled material was 14 grams, or about 54 per cent. of the theoretical. When boiled with water it was converted into the original 2-ethylmercapto-5-ethyl-6-oxypyrimidin. Analysis (Kjeldahl):

Calculated for $C_8H_{11}N_2SCl$:	Found:
N = 13.8 per cent.	13.43 per cent.

2-Ethylmercapto-5-ethyl-6-aminopyrimidin, 

This aminopyrimidin was obtained when the chlorpyrimidin above was heated in a sealed tube with alcoholic ammonia at 130°–140° C. for two hours. The excess of ammonia and alcohol was then removed by evaporation on the steam-bath. The residue was then treated with water to remove ammonium chloride. We obtained an oil that was very soluble in ether. When the ether solution was allowed to evaporate spontaneously the aminopyrimidin deposited in prismatic crystals. It was insoluble in ligroin but extremely soluble in benzene. It deposited from a mixture of these two solvents in stout prisms that melted at 74°–76° C. to an oil. Analysis (Kjeldahl):

Calculated for $C_8H_{13}N_3S$:	Found:
N = 22.95 per cent.	22.88 per cent.

2, 6-*Dioxy-5-ethylpyrimidin* (5-*Ethyluracil*), 

This pyrimidin was prepared by boiling 2-ethylmercapto-5-ethyl-6-oxypyrimidin with hydrobromic acid. It was difficultly soluble in water. One part of the pyrimidin dissolved in about 625 parts of water at 25° C. It deposited from hot water in balls of microscopic prisms that melted at about 300° C. with decomposition. Analysis (Kjeldahl):

Calculated for $C_6H_8O_2N_2$:	Found:
N = 20.0 per cent.	19.8 per cent.

5-Ethylcytosin Monohydrobromide (Anhydrous).

This salt was obtained when 2-ethylmercapto-5-ethyl-6-aminopyrimidin was boiled with hydrobromic acid. When the acid solution was allowed to stand, the hydrobromide deposited in large prisms. They did not contain water of crystallization. When heated in a capillary tube the salt began to darken at about 265° and melted at 284°–286° C. with effervescence. Analysis (Kjeldahl):

Calculated for $C_6H_9ON_3.HBr$:	Found:
N = 19.13 per cent.	19.2 per cent.

2-Oxy-5-ethyl-6-aminopyrimidin (*5-Ethylcytosin*),

```
N  =  CNH2
|     |
CO    CC2H5
|     ||
NH—  CH
```

In order to obtain this base, the monohydrobromide, described above, was dissolved in water; the bromide was removed by means of silver sulphate; the excess of silver was precipitated with hydrogen sulphide, the sulphuric acid by barium hydroxide, and then the excess of barium with carbon dioxide. The clear solution was then concentrated to a small volume, whereupon on cooling the base deposited in beautiful, slender prisms. They did not contain water of crystallization. One part of the base dissolved in about 75 parts of water at 25° C. When heated in a capillary tube it melted with effervescence at 282°–283° C. Analysis (Kjeldahl):

Calculated for $C_6H_9ON_3$:	Found:
N = 30.21 per cent.	30.03 per cent.

The base was precipitated from its aqueous solution by mercuric chloride and phosphotungstic acid. The phosphotungstate was soluble in boiling water and deposited again on cooling. When a solution of the pyrimidin base was treated with a solution of potassio-bismuth iodide a red precipitate was obtained. It was insoluble in hot water.

5-Ethylcytosin was heated with 20 per cent. sulphuric acid for six hours at 140°–150° C. When the pressure tube was examined there was no apparent decomposition. Upon concentrating on the steam-bath we did not obtain a deposit of 5-ethyluracil. The sulphuric acid was removed with barium hydroxide, and the

excess of alkali with carbon dioxide. When the neutral solution was concentrated to a small volume, the unaltered base deposited in characteristic, slender prisms that melted at 282°–283° C. with effervescence. When mixed with the original base the melting-point was not lowered.

Platinum Chloride Double Salt.

This salt was extremely soluble in water. It deposited from hot water in radiating, transparent prisms. A nitrogen determination gave the following result:

Calculated for $(C_6H_9ON_3)_2.H_2PtCl_6.2H_2O$:	Found:
N = 11.62 per cent.	11.56 per cent

The Picrate of 5-Ethylcytosin.

This salt was prepared by treating a solution of the base with picric acid. It was difficultly soluble in water. It deposited from hot water in yellow, opaque crystals. When heated in a capillary tube they decomposed at 277°–278° C.

5-Ethylcytosin Monohydrochloride (Anhydrous).

This salt was prepared by dissolving 5-ethylcytosin in dilute hydrochloric acid and allowing the solution to slowly evaporate in a desiccator over sulphuric acid. It deposited in flat prisms. They were extremely soluble in cold water and melted at 238°–240° C. with slight effervescence. Analysis (Kjeldahl):

Calculated for $C_6H_9ON_3.HCl$:	Found:
N = 23.93 per cent.	23.90 per cent.

Nitrate of 5-Ethylcytosin.

Obtained by allowing a nitric acid solution of the base to slowly evaporate in a desiccator over sulphuric acid. It deposited in stout prisms. They decomposed at about 170°–172° C. The salt was very soluble in cold water. Analysis (Kjeldahl):

Calculated for $C_6H_9ON_3.HNO_3$:	Found:
N = 27.72 per cent.	27.39 per cent.

Basic Hydrobromides of 5-Ethylcytosin.

Some of the 2-ethylmercapto-5-ethyl-6-aminopyrimidin was boiled with hydrobromic acid until ethyl mercaptan ceased to be evolved. The solution was then evaporated to dryness to remove the excess of acid. Upon adding ammonia to an aqueous solution

of the hydrobromide we obtained an immediate precipitate in the form of well developed prisms. This precipitate was difficultly soluble in water. It deposited from hot water in clusters of radiating prisms associated with diamond-shaped crystals. They decomposed at 258°–260° C. with violent effervescence. We were unable to raise this decomposition point by repeated recrystallizations from hot water. The material gave a strong test for bromine. The analytical determinations indicated that we were not dealing with a homogeneous salt, but a mixture of two hydrous, basic salts. Our determinations agreed with the calculated values for a mixture of about equal proportions of a 2:1 and a 3:1 hydrobromide.

0.3358 gram of substance lost 0.0127 gram on heating to constant weight at 100°–110° C.

For $(C_6H_9ON_3)_2.HBr.H_2O$—

Calculated: $H_2O = 4.8$ per cent.

For $(C_6H_9ON_3)_3HBr.H_2O$—

Calculated: $H_2O = 3.4$ per cent.

Average: $H_2O = 4.1$ per cent.

Found: $H_2O = 3.78$ per cent.

Nitrogen determinations in the hydrous material (Kjeldahl):

For $(C_6H_9ON_3)_2.HBr.H_2O$—

Calculated : N = 22.31 per cent.

For $(C_6H_9ON_3)_3.HBr.H_2O$—

Calculated: N = 24.41 per cent.

Average : N = 23.36 per cent.

Found : N = (1) 23.29; (2) 23.31 per cent.

Nitrogen determinations in the anhydrous material (Kjeldahl):

For $(C_6H_9ON_3)_2.HBr$—

Calculated : N = 23.39 per cent.

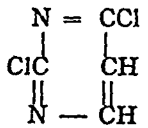
For $(C_6H_9ON_3)_3.HBr$—

Calculated : N = 25.30 per cent.

Average : N = 24.34 per cent.

Found : N = (1) 24.48; (2) 24.07 per cent.

2, 6-Dichlorpyrimidin,



This compound has been described by Gabriel.[1] He prepared

[1] *Ber. d. deutsch. chem. Gesellsch.*, xxxviii, p. 1690, 1905.

it by heating uracil with phosphorus oxychloride at 140° C. He states that it melted at 61° C. and boiled at 208.5°–209.5 ° C. at 773 mm. We had prepared the same chloride, previous to this publication, by warming 2-thiouracil[1] on the steam-bath with phosphorus pentachloride. From 35 grams of 2-thiouracil we obtained 19.2 grams of the dichlorpyrimidin boiling at 101° C. at 23 mm. In another experiment we obtained 13 grams from 20 grams of 2-thiouracil. It boiled at 198° C. at 760 mm. Our compound melted at 63° C. to a clear oil. It was soluble in alcohol, benzene, and ether. Its vapors attacked the eyes. Analysis (Kjeldahl):

Calculated for $C_4H_2N_2Cl_2$:	Found:
N = 18.79 per cent.	18.78 per cent.

[1] Wheeler and Bristol, *Amer. Chem. Jour.* xxxiii, p. 458, 1905.

THE INFLUENCE OF SUBCUTANEOUS INJECTIONS OF DEXTROSE UPON NITROGENOUS METABOLISM.

By FRANK P. UNDERHILL[1] AND OLIVER E. CLOSSON.

(*From the Sheffield Laboratory of Physiological Chemistry, Yale University.*)

(Received for publication May 13, 1906.)

It is well recognized that in diabetes there may be a greatly increased output of ammonia both absolutely and in relation to the total nitrogenous excretion. To account for this increased elimination of ammonia the theory has been advanced that the ammonia is utilized to neutralize certain unknown toxic acid products formed. The source of these acids or acid-like substances may be sought in proteid catabolism or as a result of the incomplete combustion of dextrose. That such products do originate from dextrose in diabetes is, however, by no means established.

The influence exerted by large quantities of dextrose upon proteid metabolism has recently been investigated by Scott.[2] In order to imitate as far as possible the conditions existing in diabetes, quantities of dextrose ranging from 5 to 7 grams per kilo of body weight were injected subcutaneously into dogs in nitrogenous equilibrium. A study of the distribution of the various urinary forms of nitrogen demonstrated that large quantities of injected dextrose caused a marked increase in proteid metabolism; that there was a diminution in the proportion of nitrogen built into urea, and an increase of ammonia nitrogen and of nitrogen not in the form of urea. Scott concludes from these results that "dextrose or more properly an acid derivative of dextrose under such circumstances acts like such toxic agents as phosphorus, etc., poisoning the protoplasm" and that ". . . . the administration of dextrose produces an effect similar to that induced by giving diphtheria toxin

[1] Research Fellow of the Rockefeller Institute for Medical Research.

[2] Scott, *Journ. of Physiol.*, xxviii, p. 107. 1902.

or such drugs as sulphonal which interfere with the metabolic processes."

EXPERIMENTAL.

The present investigation was undertaken for the purpose of determining more definitely what forms of nitrogen existing under the head of "non-urea" nitrogen were increased after subcutaneous introduction of large quantities of dextrose. Accordingly determinations have been made of total nitrogen of the urine and feces, and of nitrogen existing in the following urinary forms: urea, ammonia, creatinin, uric acid, and purin. The total nitrogen was estimated by the Kjeldahl-Gunning method; urea, ammonia [1] and creatinin nitrogen according to the corresponding methods of Folin [2]; uric acid nitrogen by the method of Krüger and Schmid [3]; oxalic acid by the method of Autenrieth and Barth [4]; and phosphates by titration with uranium nitrate. Unless otherwise noted dextrose-determinations were made according to the Allihn method.

The full-grown bitches, kept in the usual metabolism cages, were fed twice daily on a mixed diet, and catheterized each morning.[5] Decomposition of the urine voided during the day was prevented by the addition of toluol. In each experiment the injected fluid was distributed at several places on the body in order to facilitate absorption. The dextrose employed was that of Kahlbaum.

[1] Scott's ammonia determinations were made according to the method of Schlösing; for critique of this method see Shaffer, *Amer. Journ. of Physiol.*, viii, p. 330, 1902–03.

[2] Folin, *Amer. Journ. of Physiol.* xiii, p. 45, 1905.

[3] Krüger and Schmid, *Zeitschr. f. physiol. Chem.*, xlv, p. 1, 1905.

[4] Autenrieth and Barth, *ibid.*, xxxv, p. 327, 1902.

[5] It is well known that frequent catheterization is likely to set up cystitis in spite of precautions to keep catheters sterile, etc. Experience in this laboratory has shown that animals may be catheterized daily for months with no symptoms of bladder infection if the following procedure is observed. The catheters are kept in alcohol when not in use. After drawing off the urine and washing the bladder with sterile water, the bladder is irrigated with 50 to 100 c.c. of warm boric acid solution (saturated in the cold). After this irrigation 2 to 3 c.c. of the boric acid solution are introduced into the bladder and the catheter withdrawn. Since boric acid produces no irritation, it is retained thus keeping present in the bladder an acid medium sufficient to inhibit the growth of micro-organisms.

TABLE I. EXPERIMENT I. COMPOSITION OF URINE.

Date Oct. 1905.	Body Wt. Kilos.	Urine. Volume c.c.	Sp. Gr.	Reaction to Litmus.	Total N. Grams.	Urea N. Grams.	Ammonia N. Grams.	Creatinin N. Grams.	Uric Acid N. Grams.	Purin N. Grams.	Undetermined N. Grams.	P_2O_5 Grams.	Oxalic Acid Grams.
						Fore Period.							
25	12.2	440	1.015	acid	5.70	4.86	0.27	0.128	0.012	0.006	0.42	1.01	0.111
26	12.2	440	1.015	acid	6.00	5.13	0.27	0.133	0.013	0.005	0.45	1.16	
27	12.2	390	1.018	acid	5.88	5.06	0.25	0.128	0.011	0.005	0.43	1.14	
						Injection Period.							
*28	12.2	340	1.022	acid	†6.48	5.64	0.44	0.154	0.016	0.006	0.22	0.98	0.834
29	12.2	630	1.013	acid	6.76	5.88	0.34	0.142	0.018	0.008	0.37	0.94	
30	12.2	430	1.018	acid	6.66	5.80	0.32	0.125	—	—	—	1.43	

*Injection of 5 grams of dextrose per kilo.
†Nitrogen after subtracting 0.29 grams of proteid nitrogen, which, however, is included in the nitrogen balance.

NITROGEN BALANCE.

		Fore Period.		Sugar Injection Period.
Nitrogen intake		23.40		23.40
Nitrogen output: Urine	17.58		20.19	
Hair	0.78		0.72	
Feces	2.14		1.18	
		20.50		22.09
Balance		+2.90		+1.31

EXPERIMENT I. On October 25, 1905, a bitch of 12.2 kilos was placed upon a diet consisting of 150 grams of lean meat, 154 grams of cracker-meal and 26 grams of lard per day and containing 7.80 grams of nitrogen. On October 28th, 205 c.c. of a 30 per cent. dextrose solution (5 grams per kilo) were subcutaneously injected under antiseptic precautions. No unfavorable symptoms followed. In the urine of this day not a trace of reducing substance could be observed. Coagulable proteid corresponding to 0.29 grams of nitrogen was present. No acetone or diacetic acid could be detected. On the following days the urine was free from dextrose and proteid. The feces voided during the first days after the injection were diarrhœal in character. The distribution of the urinary forms of nitrogen is given Table I.

From the composition of the urine as shown in Table I, it is evident that the subcutaneous injection of dextrose in doses of 5 grams per kilo of body weight causes a significant increase in proteid metabolism. Accompanying the increased elimination of total nitrogen is a rise of nitrogen in the various forms distributed in the urine together with a large increase in the output of oxalic acid. It is not improbable that the latter has its origin from the introduced sugar. In Table II the percentage composition of the urine is given.

TABLE II. EXPERIMENT I. PERCENTAGE COMPOSITION OF URINE.

Date Oct. 1905	Urea N %	Ammonia N %	Urea and Ammonia N%	Creatinin N%	Uric acid N%	Purin N%	Undetermined N. %
Fore Period.							
25	85.2	4.7	89.9	2.2	0.21	0.10	7.4
26	85.5	4.5	90.0	2.2	0.21	0.08	7.5
27	86.0	4.2	90.2	2.1	0.18	0.08	7.3
Average	85.6	4.5	90.0	2.2	0.20	0.90	7.4
Injection Period.							
28	87.0	6.7	93.7	2.3	0.24	0.09	3.5
29	86.9	5.0	91.9	2.1	0.26	0.11	5.4
30	87.0	4.7	91.7	1.8	....	....	...
Average	87.0	5.5	92.4	2.1			

Inspection of this table shows that no significant rise in the proportion of any form of nitrogen found in the urine occurs.

Date Dec. 1905	Body Weight. Kilos.	Urine.										
		Volume c.c.	Sp. Gr.	Reaction to Litmus.	Total N. Grams.	Urea N. Grams.	Ammonia N. Grams.	Creatinin N. Grams.	Uric Acid N. Grams.	Purin N. Grams.	Undetermined N. Grams.	P_2O_5 Grams.
Fore Period.												
6	13.2	390	1.016	acid	6.36	5.23	0.32	0.150	0.011	0.006	0.65	0.76
7	13.3	595	1.011	acid	6.55	5.44	0.36	0.165	0.013	0.006	0.57	0.86
8	13.3	430	1.016	acid	7.11	5.93	0.37	0.157	0.016	0.006	0.63	0.89
9	13.3	460	1.015	acid	6.59	5.58	0.43	0.163	0.012	0.006	0.40	0.96
Injection Period.												
10	13.3	190	1.040	acid	7.34	6.09	0.36	0.154	0.015	0.006	0.72	0.72
11	13.5	835	1.012	acid	10.81	9.00	0.72	0.230	0.028	0.009	0.82	1.73
12	13.1	750	1.011	acid	8.16	6.79	0.53	0.152	0.025	0.008	0.66	1.56
13	13.0	430	1.018	acid	7.17	6.00	0.43	0.165	0.021	0.007	0.55	1.27
14	13.0	450	1.020	acid	7.26	6.28	0.40	0.156	0.016	0.005	0.40	1.27
15	13.3	550	1.012	acid	6.57	5.62	0.37	0.152	0.014	0.004	0.41	1.07

NITROGEN BALANCE

		Fore Period.		Sugar Injection Period.
Nitrogen intake		31.20		46.80
Nitrogen output				
Urine	26.61		47.31	
Feces	2.34		3.40	
Hair	0.54		1.07	
		29.49		51.78
Balance		+1.71		—4.98

Since the results obtained in this experiment were not in harmony with those of Scott a second trial was carried out in which the animal received a larger injection of sugar.

EXPERIMENT 2. Dec. 5, 1905. The same bitch, now weighing 13.2 kilos, was given a diet similar to that of the previous experiment and containing 7.80 grams of nitrogen per day. On Dec. 10, 310 c.c. of a 30 per cent. dextrose solution (7 grams per kilo) were injected subcutaneously with antiseptic precautions. On the few days following the injection traces of coagulable proteid could be detected in the urine. No reducing substance was present. For two days following the injection the stools were diarrhœal in character. The results of the analyses are given in Table III.

A glance at Table IV, which gives the percentages of the various urinary forms of nitrogen to total nitrogen, suffices to convince one that even the larger dose of dextrose failed to yield results in any waycomparable to those obtained by Scott.

TABLE IV. EXPERIMENT 2. PERCENTAGE COMPOSITION OF URINE.

Date Dec. 1905	Urea N %	Ammonia N %	Urea and Ammonia N %	Creatinin N %	Uric Acid N %	Purin N %	Undetermined N %
Fore Period.							
9	82.3	5.0	87.3	2.3	0.17	0.09	10.2
7	83.0	5.5	88.5	2.5	0.19	0.09	8.7
8	83.4	5.2	88.6	2.2	0.22	0.08	8.8
9	84.7	6.5	91.2	2.4	0.17	0.09	6.0
Average	83.3	5.5	88.9	2.3	0.19	0.09	8.4
Injection Period.							
10	82.9	4.9	87.8	2.1	0.20	0.08	9.8
11	83.1	6.6	89.7	2.1	0.25	0.08	7.5
12	83.2	6.5	89.7	1.8	0.30	0.09	8.0
13	83.6	6.0	89.6	2.3	0.29	0.09	7.6
14	86.5	5.5	92.0	2.1	0.22	0.07	5.5
15	85.5	5.6	91.1	2.3	0.21	0.06	6.0
Average	84.1	5.8	90.0	2.1	0.25	0.08	7.4

DISCUSSION OF RESULTS.

What is the explanation of these widely divergent results? Inspection of Scott's paper,[1] Table I, Experiment 1 (page 111),

[1] Scott, *loc. cit.*

shows that on the day of injection and for two subsequent days the urine was alkaline in reaction. This fact is strongly indicative of a severe case of cystitis probably induced by catheterization. It is common knowledge that during cystitis the quantity of ammonia is increased at the expense of the urea which circumstance coincides exactly with the result in Scott's work. Moreover, the fact that on page 115 of the paper the normal average percentage of ammonia nitrogen is given as ten per cent. confirms the indication that Scott's animals were suffering from cystitis, since in long continued experiments on many dogs in this laboratory the percentage of ammonia nitrogen has never been found to be more than half as high as that given by Scott. In the second experiment (page 111) the same conditions exist which vitiate these results also.

A study of Scott's third experiment (page 112) reveals the fact that although the urine had an acid reaction yet the increase of ammonia nitrogen amounted to ten per cent. Since the urine was acid the large increase of ammonia nitrogen cannot be accounted for by cystitis. On page 115, Experiment 3, the ammonia nitrogen during a normal period is given as 6 per cent., on the day of the injection as 16 per cent, on the first day after as 11 and on the second day after as 8 per cent. If one recalculates the percentages of ammonia nitrogen for these three days it is at once evident that an arithmetical error has been made.

	Total Nitrogen	Ammonia Nitrogen	Percentage of Ammonia Nitrogen to Total Nitrogen. Scott.	Recalculated
Day of injection	1.03	0.17	16.0	16.0
1st day after	6.86	0.13	11.0	1.8
2d day after	5.76	0.46	8.0	8.0

No possible explanation for such an error can be found from a study of Scott's work.

From the protocol given on page 113 of Scott's paper we quote the following: "On Nov. 15 at 1 P.M., the bladder was emptied by the catheter. No food was given on that day. 200 grams dextrose dissolved in 500 c.c. water were sterilized and injected subcutaneously in the left side. . . . On the following morning (Nov. 16) at 10 A.M., the bladder was emptied by the catheter. The total amount of urine excreted since the

injection was 105 c.c. . . . The urine was then diluted to 500 c.c. for further examination." It is inconceivable to the writer why on one day the animal should have been catheterized at 1 P.M. and on the next day at 10 A.M. and the urine excreted during this period taken as the entire 24 hours' elimination. To say the least such a procedure cannot be deemed accurate and consequently no correct conclusions can be drawn.

THE ABILITY OF THE ANIMAL ORGANISM TO UTILIZE DEXTROSE SUBCUTANEOUSLY INTRODUCED.

In the experiments given above and those of Scott, the very small quantities of dextrose eliminated by the kidneys are of interest in connection with subcutaneous nutrition. Many investigations have been made upon man and animals demonstrating the extent of utilization of the various carbohydrates with especial reference to the formation of glycogen. Notable among these investigations may be cited the work of Dastre,[1] C. Voit,[2] F. Voit,[3] Blumenthal,[4] and Mendel and Mitchell.[5] The limits of utilization of dextrose given subcutaneously have, however, received less attention.

To determine whether the animal employed above possessed exceptional peculiarities in this respect, the following experiments were carried out.

EXPERIMENT 3. Same bitch as in previous experiments given subcutaneously 26 c.c. of a 30 per cent. saccharose solution (0.53 gram per kilo). In the urine of the following day 74.2 per cent. was recovered (estimated polarimetrically).

EXPERIMENT 4. Same bitch as in previous experiments. On each day from Jan. 4 to Jan. 11, bitch received subcutaneously 155 c.c. of a 30 per cent. dextrose solution (3.5 grams dextrose per kilo) without more than the merest trace appearing in the urine. No reduction in the urine of the two subsequent days.

EXPERIMENT 5. Bitch of 7.7 kilos received a subcutaneous injection of 180 c.c. of a 30 per cent. dextrose solution (7 grams per kilo). The urine

[1] Dastre, *Arch. de physiol.* p. 718, 1889; p. 103, 1890.
[2] C. Voit, *Zeitschr. f. Biol.* xxviii, p. 245, 1891.
[3] F. Voit, *Münch. med. Wochenschr.* p. 717, 1896.
[4] Blumenthal, *Beitr. z. chem. Physiol.* vi, p. 329, 1905.
[5] Mendel and Mitchell, *Amer. Journ. of Physiol.*, xiv, p. 239, 1905.

of the following 24 hours contained 1.63 grams dextrose; no reduction on the two subsequent days.

Experiment 6. Bitch of 10.6 kilos was injected subcutaneously with 248 c.c. of a 30 per cent. dextrose solution (7 grams per kilo). The urine of the following day gave merely a faint reduction; no reduction on the two subsequent days.

From the above protocols it is seen that the experimental animal employed in the first experiments is not peculiar with respect to its ability to utilize large quantities of dextrose when introduced subcutaneously. In order to demonstrate whether dextrose given intraperitoneally would be equally well retained the following experiment was carried out.

Experiment 7. Bitch of 14.5 kilos received an intraperitoneal injection of 244 c.c. of a 30 per cent. dextrose solution (5 grams per kilo). The 24 hours urine contained 1.95 grams of dextrose. The urine of the subsequent day contained 1.04 grams of dextrose. On the following days no reducing substance could be demonstrated in the urine.

The possibility was suggested that part of the dextrose might be eliminated by way of the intestine, but after the subcutaneous injection of 310 c.c. of a 30 per cent. dextrose solution (7 grams per kilo) the feces for the four following days contained only one gram of reducing substance from which osazones could not be formed.

Experiments were also carried out to determine whether a herbivorous animal behaves in a way similar to the carnivora toward subcutaneous injections of dextrose. For this purpose the rabbit was selected.

Experiment 8. Rabbit of 2.5 kilos received subcutaneously 42 c.c. of a 30 per cent. dextrose solution (5 grams per kilo). Urine for two days following the injection was collected by compression of the bladder and found to be free from reducing substances.

Experiment 9. Rabbit of 2.1 kilos. 50 c.c. of a 30 per cent. dextrose solution (7 grams per kilo) were introduced subcutaneously. Only the merest trace of dextrose appeared in the urine on the three subsequent days.

That the ability of the body to utilize dextrose after subcutaneous injection is large is indicated by the results of the foregoing experiments. The observations further suggest that

such injections may play an important rôle in connection with subcutaneous nutrition from a clinical standpoint.

CONCLUSIONS.

No evidence has been obtained that large quantities of dextrose subcutaneously injected exert a toxic action resulting in changes in the urinary forms of nitrogen. The conclusion of Scott that dextrose may thus act like phosphorus, etc., by poisoning the protoplasm has not been substantiated.

The results of Scott are attributed in part to ammoniacal fermentation induced in the urine of his experimental animals, and also to errors which have crept into his work.

The present experiments also demonstrate the great power of the animal body to utilize dextrose given subcutaneously.

THE CLEAVAGE PRODUCTS OF VITELLIN.

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(Received for publication, May 24, 1906.)

Biologists have always taken considerable interest in vitellin, owing to its relation to hæmoglobin and to nuclein. Hence, it is natural that the substance also attracted the attention of chemists. Vitellin is distinguished from other proteids on account of its acid properties. The component upon which the acid properties depends has attracted our attention for several years. It seemed desirable to study the relation of the proteid entering into the molecule of vitellin to other proteids, and especially to those composing true nucleoproteids. Moreover, an effort to compare the composition of the proteid in vitellin with that of vitellinic acid was undertaken.

The present communication refers to the composition of vitellin. In a later report the results of a study on vitellinic acid will be given.

The substance employed in the course of this work was prepared in the same manner as the one used in our previous work.[1] It was once reprecipitated and then washed by decantation. The hydrolysis was accomplished by boiling with four parts of strong hydrochloric acid and the amino-acids were studied by means of Fischer's ester method.

The hexon bases were separated from the part remaining after the extraction of the esters by means of ether. However, to render the course of analysis possible it was thought desirable to modify somewhat the process of Fischer. The transformation of the hydrochloride of the esters into the free esters is accomplished, according to Fischer, by means of potassium carbonate and sodium hydrate. This naturally results in saturating with inorganic salts the part remaining after the removal of the amino-acids; and this in its turn makes the analysis of this fraction very troublesome. However, if dry barium oxide and a solution of

[1] *Zeitschr. f. physiol. Chem.*, xxxii, p. 281, 1901.

barium hydrate are employed instead of the alkalies mentioned the removal of the alkali is accomplished without difficulty.

Three hundred grams of vitellin and two liters of hydrochloric acid wer heated in a reflux condenser for six hours. The product of hydrolysi was esterified in the usual manner by means of dry hydrochloric acid an absolute alcohol. The operation was repeated three times. The exces of hydrochloric acid and of alcohol was then removed under diminishe pressure. The transformation of the hydrochlorides into the free ester was accomplished in the same manner as was done by Fischer, with th exception that dry barium oxide was employed instead of potassiun carbonate and a concentrated solution of barium hydroxide instead o sodium hydrate.

The residue from the free esters was esterified a second time with dr hydrochloric acid and absolute alcohol. To make this possible th barium was removed quantitatively by means of sulphuric acid.

The two ethereal extracts containing the free esters were combine and, after the removal of the ether, subjected to fractional distillatio at 15 mm. pressure.

The following fractions were obtained:

Up to 60°C........	15.2 grams
60 " 80°C.........	17.0 "
80 " 90°C.........	15.5 "
95 " 105°C.........	2.9 "
105 " 165°C.......	21.6 "
Total........	72.2 grams

This yield of esters should be regarded as the minimal, sinc the work was done during the late summer months when it wa impossible to obtain a vacuum by the water pump lower tha 15 millimeters; furthermore, considerable time elapsed betwee the first and second esterification. It is possible that the ester of the first extraction had suffered some condensation.

Fraction up to 60°C. From this fraction glycocoll and alanin were obtained. For the separation of glycocoll the amino-acid of this fraction were again esterified by means of alcohol and dr hydrochloric acid. About 0.3 gram of the hydrochloride o glycocoll ethylester was obtained. The substance was onc recrystallized out of absolute alcohol. Its melting point wa 144°C.

The filtrate from glycocoll was diluted with water and heate in a reflux condenser for three hours. The alcohol was then removed by evaporation and the hydrochloric acid by means of

silver sulphate. While this work was in progress one of us (Levene) made the observation that phosphotungstic acid may be employed for the separation of certain amino-acids. Thus the solution of the amino-acids of this fraction containing sulphuric acid was treated with a concentrated solution of phosphotungstic acid (4 parts of acid to 1 of water), so long as an oily precipitate was formed. The supernatant liquid was decanted and treated with an excess of the same reagent. A crystalline phosphotungstate was obtained. Both glycocoll and alanin form a crystalline phosphotungstate. However, according to the observation of one of us (Levene) glycocoll forms an insoluble picrate while alanin does not. The crystalline phosphotungstate obtained from this fraction was decomposed in the usual manner, concentrated to a very small volume, and tested with an alcoholic solution of picric acid. On standing, no picrate of glycocoll could be obtained. On treatment with alcohol and ether there resulted a precipitate having the composition of alanin.

0.1285 gram of the substance gave on combustion 0.1896 gram CO_2 and 0.0975 gram H_2O.

Calculated for $C_3H_7NO_2$:	Found:
C = 40.45 per cent.	40.24 per cent.
H = 7.87 " "	8.40 " "

The yield was about 0.500 gram.

Fractions 60–80° and 80–90°C. The analysis of these and of the following fractions was completed before the observation on the behavior of phosphotungstic acid toward amino-acids was made. It was, therefore, carried out by the method of Fischer. The two fractions were saponified by boiling with water and evaporated to dryness. The residues were extracted with boiling absolute alcohol. The part insoluble in alcohol was recrystallized from water. The substance thus obtained had all the properties of leucin.

The substance recrystallized a second time had the following composition:

0.1529 gram of the substance was employed for a Kjeldahl nitrogen estimation. It required for neutralization 12 00 c.c. of $\frac{N}{10}$ H_2SO_4.

Calculated for $C_6H_{13}NO_2$:	Found:
N = 11 02 per cent.	10.98 per cent.

The total yield of leucin was 10 grams.

From the filtrates from the leucin an attempt was made to obtain the copper salts of aminovalerianic and of aminobutyric acids. The salts obtained did not, however, possess the composition of the acids sought.

The alcoholic extracts of these fractions were combined and evaporated to dryness; the residue again extracted with absolute alcohol and the solution evaporated. The operation was repeated three times. The final residue was allowed to stand in a vacuum desiccator over sulphuric acid. After drying for two days in this manner the extract weighed 12.0 grams. It was regarded as crude prolin. In order to identify the substance it was transformed into the inactive acid by heating with barium hydroxide for five hours in an autoclave at 150°C. After cooling, the barium was removed by means of sulphuric acid and the filtrate evaporated to dryness. The residue was extracted with absolute alcohol and the alcoholic extract employed for the preparation of the copper salt of the inactive prolin. Three grams of the salt recrystallized out of water were obtained.

0.5874 gram of the substance gave 0.0626 gram H_2O.
0.1300 gram of the substance gave 0.0320 gram CuO.

Calculated for $(C_5H_8NO_2)2Cu + 2H_2O$:	Found:
H_2O = 10.99 per cent.	10 66 per cent.
Cu = 19.27 " "	19.62 " "

Fraction 95–105°C. Leucin was obtained from this fraction. The yield was 1 gram.

Fraction 105–165°C. From this fraction aspartic and glutamic acids and phenylalanin were isolated. For this purpose the esters were dissolved in ether and the first two acids extracted from the ethereal solution by means of water.

Phenylalanin ester was saponified by means of hydrochloric acid and the hydrochloride of the substance thus obtained transformed into the free acid by means of ammonia. For identification it was recrystallized out of water and analysed. The yield was 2.9 grams.

0.1194 gram of substance gave on combustion 0.2850 gram CO_2 and 0.0700 gram H_2O.

Calculated for $C_9H_{11}NO_2$:	Found:
C = 65.45 per cent.	65.09 per cent.
H = 6.66 " "	6.52 " "

The fraction containing aspartic and glutamic acids was saponified with barium hydroxide. The first substance separated in the form of the basic barium salt. The yield was 1.7 grams. For analysis it was recrystallized out of water.

0.1700 gram of the substance gave on combustion 0.2262 gram CO_2 and 0.080 gram H_2O.

Calculated for $C_4H_7NO_4$:	Found:
C = 36.09 per cent.	36.25 per cent.
H = 5.26 " "	5.68 " "

The filtrate from the basic barium salt of aspartic acid was employed for the separation of glutamic acid. For this purpose the barium was removed quantitatively, the solution evaporated to dryness, the residue taken up in concentrated hydrochloric acid, and the hydrochloride of the substance allowed to crystallize. This was transformed into free glutamic acid in the usual manner. The yield was 3.3 grams.

0.1352 gram of the substance gave on combustion 0.1985 gram CO_2 and 0.0147 gram H_2O.

Calculated for $C_5H_9NO_4$:	Found:
C = 40.81 per cent.	40.95 per cent.
H = 6.12 " "	6.14 " "

Tyrosin. The residue from the esters was employed for the separation of tyrosin and of the hexon bases. It was dissolved n a considerable amount of water, the barium was then removed quantitatively, and the filtrate from the barium sulphate, which still contained hydrochloric acid, was heated for several hours on a water-bath. The excess of hydrochloric acid was then removed by means of lead oxide; the excess of lead removed by sulphuretted hydrogen. The filtrate from the lead sulphide was concentrated to a small volume. On concentration, tyrosin crystallized out. The yield was about 1 gram.

For analysis the substance was recrystallized out of acetic acid.

0.1379 gram of the substance was employed for a Kjeldahl nitrogen estimation. It required for neutralization 7.5 c.c. of $\frac{N}{10}$ H_2SO_4.

Calculated for $C_9H_{11}NO_3$:	Found:
N = 7.73 per cent.	7.50 per cent.

Hexon Bases. The filtrate from tyrosin was acidified with sulphuric acid and treated with a 50 per cent. solution of

phosphotungstic acid. This caused the formation of a gummy precipitate (Ppt. I). When further treatment with the reagent ceased to bring down a precipitate of that nature, the supernatant liquid was removed by decantation and the treatment with the reagent was continued. A second precipitate (Ppt. II) of a finely granular character was formed.

Each of the precipitates was decomposed in the usual manner, and was further treated for hexon bases by the process devised by Kossel and Kutscher. The first precipitate yielded no histidin. From the arginin fraction an amorphous silver salt was obtained, which possessed all the properties of a substance obtained by one of us (Levene)[1] from a similar fraction of the products of hydrolytic cleavage of heteroalbumose. The alcoholic ethereal mother liquid from the amorphous silver salt, on long standing, deposited on the walls of the vessel a granular substance resembling arginin silver nitrate.

The lysin fraction yielded about 6 grams of lysin picrate. For identification the substance was once recrystallized out of water.

0.1797 gram of the substance gave on combustion 29 5 c.c. of N (over 50 per cent. KOH) at $t = 22°C.$ and $p = 760.0$ mm.

Calculated for $C_6H_{14}N_2O_2.C_6H_3N_3O_7$:	Found:
$N = 18.65$ per cent.	18.59 per cent.

In the second precipitate the histidin fraction was larger than in the first one. However, the yield of the dichloride was so small that it could not be purified for analysis. The presence of histidin in the molecule of vitellin is made certain by the fact that it was isolated by the writers from the products of hydrolysis of vitellinic acid.

The arginin fraction yielded 7.5 grams of the silver nitrate salt of arginin.

0.1200 gram of the substance once recrystallized gave 0.0318 gram of Ag.

Calculated for $C_6H_{14}N_4O_2.HNO_3 + AgNO_3$:	Found:
Ag. $= 26.54$ per cent.	26.50 per cent.

No picrate could be obtained from the lysin fraction. It was

[1] This Journal, i, p. 45, 1905.

also impossible to obtain any substance in a crystalline form from this fraction.

Calculated for 100 grams of vitellin, the yield of the constituents was as follows:

Glycocoll	trace	
Alanin	0.16	grams
Leucin	3.30	"
Prolin	4.00	"
Aspartic acid	0.6	"
Glutamic acid	1.00	"
Phenylalanin	1.00	"
Tyrosin	0.400	"
Histidin	trace	
Arginin	1.20	"
Lysin	2.40	"

It has already been stated that the yield of the various constituents indicates only the minimal quantities present in the molecule. But the high proportion of prolin to other amino-acids is especially noteworthy. This is very significant, since vitellin furnishes the material for the production of hæmoglobin.[1]

[1]Some time after the work described in this paper had been completed a communication on the same subject by Hugounenq appeared in the *Journal de physiologie et de pathologie générale* (viii, p. 209, 1906). In view of the fact that in the present communication some modifications of Fischer's process are described, and also in view of the quantitative differences existing between some of our results and those of Hugounenq. we have concluded to publish our findings.

ON SULPHATE AND SULPHUR DETERMINATIONS.

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(Received for publication, February 22, 1906.)

In the article by Otto Folin[1] under the above title, we find the following in the section entitled "Gooch Crucibles *versus* Filter Paper in Sulphate Determinations" (p. 148): "It has indeed been pointed out that some error may creep in in connection with the burning of the filters, but this error has been explained as a reduction of sulphate to sulphide. Occasionally this may happen, but not often. It is seldom that barium sulphate precipitates gain in weight after treatment with a drop of sulphuric acid and reheating. The mechanical loss of notable quantities of barium sulphate occurs, however, nearly every time."

The writer's experience is in entire harmony with the last sentence of the above quotation, but not with the remainder of that section. When the filters containing the barium sulphate precipitates are "burned" in an open crucible there is nearly always a mechanical loss. The writer has therefore resorted to the method of heating the filter gently in a closed crucible until gases no longer escape, the crucible lid is then moved slightly to one side to leave openings for the entrance and exit of air, and the charred filter is completely oxidized. In this way very little mechanical loss is experienced. For several years the writer and his students have been using Gooch crucibles with asbestos mats for collecting barium sulphate, calcium sulphate, silver chloride, etc., and have found them very serviceable. The crucible can be dried by heating it with a Roger's burner, or in a slightly larger iron or copper crucible with a lid; in the latter case, the Gooch crucible must stand on a clay triangle or on a porcelain plate.

[1]This Journal, i, p. 131, 1906.

The idea that barium sulphate is not often reduced by the heated carbon or gases, and at most to only a slight extent is a serious error and unfortunately one that has widespread acceptance. Barium sulphate is reduced to barium sulphide under the conditions generally used, and this reduction may even reach nearly the theoretical value. When the residue is treated with the dilute sulphuric acid a strong odor of hydrogen sulphide is noticeable. Furthermore, it will be recalled that calcium sulphide is made commercially by heating a mixture of calcium sulphate and finely divided coal. In order to impress this reduction phenomenon thoroughly upon their minds, the writer has often made his students in quantitative analysis determine the weight of the barium sulphate immediately after burning the filter, and then again after treating this with sulphuric acid; the increase in weight is very often ten per cent. of the weight of the barium sulphate. Of course when the main portion of the barium sulphate is removed from the dried filter before the latter is burned, the error involved is not large even when the barium sulphide is not converted into barium sulphate. But when the entire amount of barium sulphate is burned in the filter, as is often the case when the quantity is small, and as is very frequently the case in technical "rapid method" laboratories, where the *wet* filter is put into the crucible to be dried and burned in the same operation, the amount of reduction may be large. And the deplorable feature is that in many of these technical laboratories the residue is not subsequently treated with sulphuric acid, or at most only once. Their results then are always too low, unless enough of other salts is included in the barium sulphate to make up for the error.

The following experiments, however, will abundantly illustrate the point that barium sulphate is very easily reduced under the conditions used and that the sulphide is not oxidized back into barium sulphate completely, even when it is heated for considerable periods of time. The figures representing the amount of reduction observed are lower than the actual amount of reduction for the reason that some of the barium sulphide is oxidized back into barium sulphate during the reduction process and part of the barium sulphide is probably converted

into barium carbonate by carbonic acid. Hydrogen sulphide is constantly evolved from the crucible when it is open to the moisture and carbon dioxide of the atmosphere. The experiments further clearly show that the barium sulphide is not easily converted completely into barium sulphate by sulphuric acid. Just as water penetrates the crystals of barium sulphate and dissolves occluded salts only with difficulty, so the sulphuric acid penetrates the crystals and converts the sulphide into sulphate only very slowly. A coating of sulphate formed around the sulphide protects the latter from the action of the acid and it is often necessary to heat the residue several times with concentrated sulphuric acid to secure a constant weight. Even then it is doubtful whether all of the sulphide has been acted upon, and the writer feels that the low results often obtained by the barium sulphate method are due to this error as well as to the error from mechanical loss. In the following experiments the ignitions were made in a closed platinum crucible with a Roger's burner.

The sugar and filter papers were burned slowly to avoid mechanical losses by the issuing gases. The sulphuric acid used left no residue when evaporated. The per cent. of reduction was determined from the loss in weight and the weight of the total barium sulphate.

Experiment I.

Pure ignited barium sulphate	1.1021	grams
After burning with one gram of cane sugar	0.9666	"
Observed reduction, 12.30 per cent	0.1355	"
After heating 30 minutes	1.0158	"
After heating 60 minutes	1.0596	"
After treating with 3.0 c.c. dilute sulphuric acid	1.1001	"
After treating with 3.0 c.c. concentrated sulphuric acid, (weight still 0.0015 gram too low)	1.1006	"

This experiment shows (1) that barium sulphate is very easily reduced; (2) that barium sulphide is not easily oxidized to barium sulphate by hot oxygen, and (3) that barium sulphide is not converted completely into barium sulphate by two treatments with sulphuric acid.

EXPERIMENT II.

After slowly igniting the wet filter paper containing all of the barium sulphate..	2.0755 grams
After heating once with 3.0 c.c. of dilute sulphuric acid ..	2.2465 "
After heating with 3.0 c.c. of concentrated sulphuric acid	2.2475 "
After heating twice with 3.0 c.c. of concentrated sulphuric acid	2.2485 "
After heating three times with 3.0 c.c. of concentrated sulphuric acid	2.2495 "
After heating four times with 3.0 c.c. of concentrated sulphuric acid	2.2500 "
Observed reduction, 7.76 per cent	0.1745 "

This experiment shows (1) how difficult it is to change a large amount of barium sulphide into barium sulphate by means of sulphuric acid, and (2) how easily barium sulphate determinations can be systematically low on account of reduction when the wet filter is dried and burned in one operation.

EXPERIMENT III.

After igniting the dry filter paper containing all of the barium sulphate and heating 30 minutes after the carbon was completely burned	0.4313 grams
After evaporating with 3.0 c.c. of dilute sulphuric acid...	0.4487 "
After evaporating with 3.0 c.c. of concentrated sulphuric acid	0.4495 "
After evaporating twice with 3.0 c.c. concentrated sulphuric acid	0.4495 "
Observed reduction, 4.05 per cent	0.0182 "

This experiment shows (1) that with smaller quantities of barium sulphate the amount of reduction is still considerable, and (2) that three evaporations with sulphuric acid were necessary to secure constant weight.

The uncertainty of results obtained by the barium sulphate method has caused the writer to avoid its use whenever possible and to employ others, the accuracy of which was carefully tested.

In the standardization of approximately normal solutions of pure sulphuric acid (and hydrochloric acid) the following method, devised by the writer, has proved to be the most advantageous. Sodium bicarbonate was crystallized twice and was then free from chlorides, sulphates, and silicates. Approxi-

mately 4.12 grams of this were introduced into a tall beaker, covered with a watch glass with a hole in the centre, and titrated with the necessary volume of the acid (nearly 50.0 c.c.), methyl orange being the indicator. The solution was then brought to boiling in the beaker to expel the carbon dioxide and was then evaporated to dryness and ignited in a weighed platinum dish. Another similar platinum dish was used as a tare in all the weighings. From the weight of the residual sodium salt and the volume of acid used the strength of the acid solution was easily determined. From 0.005 c.c. to 0.01 c.c. of normal acid solution is sufficient to give the color change with methyl orange; the error of titration need not then be more than 0.02 per cent., and the weighing error is not more than 0.01 per cent. Special experiments showed that when 50.0 c.c. of water, containing one drop of 1:2000 methyl orange solution, was treated with pure carbon dioxide, the pink color produced was discharged by 0.05 to 0.50 c.c. $\frac{N}{10}$ potassium hydroxide solution. A similar solution of 50.0 c.c. water, one drop methyl orange solution, and 0.50 c.c. $\frac{N}{10}$ potassium hydroxide solution was not changed to the faintest pink when saturated with carbon dioxide. It is obvious then that in titrating carbonates in the presence of methyl orange, the solution must be titrated with acid till the color is just as deep a shade of pink as that of a similar volume of water saturated with carbon dioxide and containing the same amount of indicator; otherwise the solution will still contain a small amount of carbonate or bicarbonate of the metal not neutralized by the strong acid. These experiments indicate just how sensitive methyl orange is to the very weak carbonic acid.

It is obvious that the method can be used for the determination of the strength of the solution of any acid whose sodium salt can be dried. The titration error is larger for weaker acids, and should be determined for each case. The acids used for making the standard solutions must be chemically pure.

The accuracy of the method is shown by the following data which were kindly furnished by Mr. R. F. Brunel. A solution of hydrochloric acid known to be 0.99987 normal was used.

C.c. of Acid Used.	Weight of NaCl.	Normal Strength.
30.30	1.7730	1.00020
30.50	1.7833	0.99937
47.24	2.7629	0.99976

Average, 0.99972
Theory, 0.99987
Error, 0.015 per cent.

A solution of sulphuric acid, made up by titration methods through two other solutions to be exactly normal, was analyzed by this method and also checked by a barium sulphate determination.

C.c. of Acid Used	Weight of Na_2SO_4	Normal Strength
45.33	3.2288	1.0021
47.32	3.3723	1.0026

Average, 1.0023

C.c. of Acid Used	Weight of $BaSO_4$	Normal Strength
20.68	2.4170	1.0013

The two values obtained by the new method agree very closely, namely to within 0.05 per cent. As the titration errors involved in standardizing the normal sulphuric acid aggregated 0.20 per cent., and as the error in the barium sulphate determination was probably 0.10 per cent., the value 1.0023 N obtained by the new method agrees very closely with the values obtained by the other methods, and is probably not more than 0.03 per cent. from the correct concentration.

The writer feels that the method can be relied upon, with careful work, to give results which are accurate to approximately 0.03 per cent. In a similar manner the exact strength of a solution of pure sodium or potassium hydroxide, or carbonate, can be determined by evaporating an exact volume, approximately 50.0 c.c., with an excess of pure hydrochloric acid, and weighing the residual salt. The weight of the chloride obtained and the volume of alkaline solution used give data

for determining the strength of the alkaline solution. If the alkali, however, contains silicates, sulphates, or chlorides, etc., the method cannot be used.

The writer has found Wildenstein's volumetric method to be of great service in estimating sulphates in mixtures. $\frac{N}{10}$ solutions of barium chloride, sodium sulphate, and potassium chromate are convenient for general work. The experimenter must first become familiar with the color changes. The writer has checked this method against the barium sulphate and calcium sulphate methods and finds it accurate to within 0.20 per cent., the exactness depending upon the operator. The following analyses give an idea of how accurately different titrations check:

Exp. 1.—Solution of sodium sulphate: 15.00 c.c. = 14.25, 14.16, 14.29, 14.19, or an average of 14.22 c.c. $\frac{N}{10}$ $BaCl_2$ solution.

Exp. 2.—Solution of potassium sulphate; 20.00 c.c. = 10.04, 10.05, 10.07, 10.08, or an average of 10.06 c.c. $\frac{N}{10}$ $BaCl_2$ solution.

Exp. 3.—Solution of ammonium sulphate; 15.00 c.c. = 8.47, 8.41, 8.45, 8.43, or an average of 8.44 c.c. $\frac{N}{10}$ $BaCl_2$ solution.

Exp. 4.—Solution of magnesium sulphate; 15.00 c.c. = 15.19, 15.21, 15.20, or an average of 15.20 c.c. $\frac{N}{10}$ $BaCl_2$ solution.

Exp. 5.—Solution of potassium sulphate; 15.00 c.c. = 6.69, 6.58, 6.70, 6.72, 6.76, 6.76, 6.67, 6.69, or an average of 6.70 c.c. $\frac{N}{10}$ $BaCl_2$ solution.

Exp. 6.—Solution of ammonium sulphate; 15.00 c.c. = 14.77, 14.78, 14.72, 14.76, 14.71, 14.70, or an average of 14.74 c.c. $\frac{N}{10}$ $BaCl_2$ solution.

Exp. 7.—Solution of sodium sulphate; 12.00 c.c. = 11.34, 11.40, 11.42, 11.30, 11.31, 11.38, or an average of 11.36 c.c. $\frac{N}{10}$ $BaCl_2$ solution.

Exp. 8.—Solution of potassium sulphate; 13.50 c.c. = 12.73, 12.74, 12.72, 12.71, or an average of 12.725 c.c. $\frac{N}{10}$ $BaCl_2$ solution.

Exp. 9.—Solution of sodium sulphate; 10.00 c.c. = 10.27, 10.23, 10.23, 10.24, 10.26, 10.22, or an average of 10.24 c.c. $\frac{N}{10}$ $BaCl_2$ solution.

Exp. 10.—Solution of barium chloride; 10.00 c.c. = 9.84, 9.84, 9.82, 9.82 or an average of 9.83 c.c. 1.024 $\frac{N}{10}$ Na_2SO_4 solution.

Instead of the barium sulphate method the writer often uses the method in which calcium sulphate is precipitated from a 60 to 75 per cent. alcohol solution. This can be employed only when no other substance precipitates out with the calcium sulphate, and can be used for determining calcium as well. Often a knowledge of the conditions present will enable the operator to choose the volume of solution necessary to retain in solution all of the salts except the calcium sulphate. The

method of procedure is the following. The water solution of the sulphate (or calcium salt) is diluted with two or three volumes of alcohol, and warmed. To this solution is added a concentrated solution of calcium chloride (or sulphuric acid), whereupon the calcium sulphate precipitates out as a feathery mass. The solution is allowed to cool two hours and is then filtered on a Gooch crucible or on a filter wet with alcohol. In either case the precipitate is washed well with absolute alcohol and dried quickly at 110° C. If the precipitate is collected in a Gooch crucible this is dried at a faint red heat. When a filter paper is used, the precipitate can be practically completely removed from the filter paper without the least difficulty. The filter is then burned as above, and treated with sulphuric acid till constant in weight; the main portion of the calcium sulphate is then added to the crucible, which is then heated to faint redness at the upper edge with a Roger's burner.

The following experiments illustrate how accurately the determinations check. The first analysis gives an idea of the exactness of the method.

Exp. 1.—Calcium sulphate solution: (1) 98.86 c.c. evaporated to dryness and ignited gave 0.2116 gm. $CaSO_4$.—(2) 99.05 c.c. solution, treated with two volumes alcohol, gave 0.2120 gm. $CaSO_4$. Calculated for 100.0 c.c. (1) 0.2140 gm., (2) 0.2140 gm. $CaSO_4$.

Exp. 2.—Calcium nitrate solution: (1) 15.06 c.c. gave 0.2695 gm. $CaSO_4$.—(2) 15.31 c.c. gave 0.2743 gm. $CaSO_4$. Calculated for 10.00 c.c. (1) 0.17895 gm., (2) 0.17916 gm. $CaSO_4$.

Exp. 3.—Sodium sulphate solution: (1) 13.42 c.c. gave 0.1037 gm. $CaSO_4$.—(2) 14.90 c.c. gave 0.1149 gm. $CaSO_4$. Calculated for 20.00 c.c. (1) 0.1545 gm., (2) 0.1542 gm. $CaSO_4$.

Exp. 4.—Calcium nitrate solution: (1) 20.06 c.c. gave 0.1398 gm. $CaSO_4$.—(2) 19.99 c.c. gave 0.1394 gm. $CaSO_4$. Calculated for 20.00 c.c. (1) 0.1394 gm., (2) 0.1395 gm. $CaSO_4$.

CONCLUSIONS.

1. Barium sulphate is very easily reduced to barium sulphide, and the amount may often be half the theoretical value. Several evaporations with sulphuric acid may be necessary to change barium sulphide into barium sulphate.

2. A new method for standardizing solutions of pure hydro-

chloric acid, sulphuric acid, alkalies, and alkali carbonates, gives very accurate results

3. The titration method of Wildenstein for sulphates, or for barium, gives fairly accurate results. The method for the estimation of calcium, and of sulphates, involving the precipitation of calcium sulphate from alcoholic solutions, gives fairly accurate results.

ON THE DETECTION OF FORMALDEHYDE IN MILK.

(Preliminary Contribution.)

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(Received for publication, February 26, 1906.)

When milk containing a trace of formaldehyde is mixed with an equal volume of water and four volumes of concentrated sulphuric acid, in which there is a small amount of a ferric salt, the solution soon acquires a violet color (Richmond and Boseley's modification of Hehner's test). If comparison tubes are simultaneously made from milk containing known quantities of formaldehyde, a comparison of the color in the milk solution under investigation with that of the standard solutions will give roughly the concentration of the formaldehyde in the milk which is to be analyzed. The test is so sensitive that one part of formaldehyde in 200,000 of milk can be readily detected, but the test cannot be used for concentrated solutions.

In the study of this analytical method, three questions are of great theoretical and practical interest: (1) What constituent, or constituents, of the milk is required to give the color? (2) What is the nature of the colored compound? (3) Do any other substances, not constituents of milk, give this same test for formaldehyde, and do other aldehydes behave like formaldehyde? Obviously the first and third questions can be far more easily answered than the second one, and to the study of these the present paper is devoted. As time permits, the work will be forwarded and it is hoped that further light on this interesting problem can be secured.

In the study of the first question the various constituents of milk—casein, lactalbumin, lactose, milk fat, the mineral matter—were subjected to this modification of Hehner's test. That is, each substance was mixed with a small volume of 1:5000 formaldehyde and to this was added some of the sulphuric acid. Only the casein and the lactalbumin gave the violet color, and the

success of Hehner's test depends upon the presence of these two substances in the milk. Furthermore, there is some kind of a quantitative relationship between the intensity of the color and the amount of casein and lactalbumin in the milk. Of two samples of milk containing the same per cent. of formaldehyde and different amounts of casein, the one having the larger percentage of casein will give the more intense color. This can be very readily shown as follows: when to 5.0 c.c. of milk, containing 1 : 50,000 formaldehyde, 0.25 gram of commercial casein is added, the color produced by Hehner's test is considerably more intense than that produced by the check test on the milk. If 0.50 gram of casein is added to another 5.0 c.c. of the same sample of milk the color is still more intense, and any increase in the amount of casein added to the milk causes a deepening of the color, up to a certain limit. The point of interest is, then, that the intensity of the color depends not only upon the amount of formaldehyde in the milk but also upon its richness in casein and lactalbumin. The delicacy of Hehner's test *as a test for milk or formaldehyde* is shown by the fact that a mixture of 0.005 c.c. of milk, 0.005 c.c. of formaldehyde solution (1:5000), and two drops of the sulphuric acid give a very distinct violet color. As these volumes of milk and formaldehyde solution contain 0.0002 gram of casein and 0.000001 gram of formaldehyde, it is seen that the test is very delicate for both of these substances.

In the study of the third question, it was found that of all the other aldehydes tested (acetaldehyde, paraldehyde, chloral-hydrate, benzaldehyde, cuminal, vanillin) only the vanillin gave the violet color in milk and sulphuric acid. As the degree of concentration of the aldehyde is an important factor, other aldehydes, ketones, etc., and the experimental conditions will be studied further.

Not only does casein give the violet color with formaldehyde and the sulphuric acid, but all other proteids tested give the same color. On the other hand lactose, glucose, cane sugar, starch, butter, monamino acids, urazoles, and many other substances do not give the slightest response to Hehner's test. This work will be reported in detail later, but it may be stated briefly that only the complex proteids seem to form the colored compounds.

Of the various proteids investigated, the globulin from squash-seed, kindly furnished by Professor T. B. Osborne, gave the most intense violet color. In fact, the depth of the color is so great that the writer finds it very valuable as an aid in detecting the presence of formaldehyde in milk in concentrations less than 1:250,000. It is well known that Hehner's test fails to reveal the presence of formaldehyde in milk in concentrations less than 1:250,000. But we are continuing researches on changes in milk containing even smaller quantities of formaldehyde, and it seemed desirable to have a test whereby we might be able to detect even the minutest traces of this preservative in milk.

The following method is very satisfactory. Twenty c.c. of the milk containing the formaldehyde are distilled from a 50.0 c.c. side-neck flask; the side-neck has a small condenser around it and a small short test-tube, containing 0.01 gram of globulin and 0.25 c.c. of water, is placed at the end of the side-neck so that the escaping vapors bubble through the globulin solution. When 0.5 c.c. of liquid is collected in the test-tube, some of the sulphuric acid is added so as to form a layer under the globulin solution; when the two layers are slightly mixed the violet zone is formed at once. This test is so delicate that 20.0 c.c. of milk containing formaldehyde in the concentration 1:1,000,000 easily yield four successive fractions that give this violet color. Milk containing formaldehyde in the concentration 1:3,000,000 gives one fraction that shows the violet color very plainly. By fractional distillations the formaldehyde in almost infinitesimally dilute solutions could probably be easily detected.

The nature of the colored compounds is not at once apparent. It is probable that formaldehyde enters into combination with the amide groups of the proteids, and that these reactions are reversible.

$$R_2NH + CH_2O \rightleftarrows R_2NCH_2OH$$ [1]

$$RNH_2 + CH_2O \rightleftarrows RN{=}CH_2 + H_2O$$

$$2\ RNH_2 + CH_2O \rightleftarrows (RNH)_2CH_2 + H_2O$$

[1] Bailey and Acree, *Ber. d. deutsch. chem. Gesellsch.*, xxxiii, p. 1520; Einhorn, *Ann. d. Chem.*, cccxliii, p. 207

This view is substantiated by the following facts. The formaldehyde must react with the proteid before the sulphuric acid is added, or no color results. The fact that the formaldehyde, even in extremely small quantities, can be distilled out of the milk shows that the reaction is reversible and that the formaldehyde and proteid are in only very loose combination. Finally, the fact that the addition of a larger amount of casein to milk containing only a trace of formaldehyde causes a deeper color by Hehner's test shows that the proteid-aldehyde compound is formed in larger amount by the presence of the larger quantity of casein. This would be expected if the reaction were reversible and if only a part of the formaldehyde is in combination with the proteid. We assume that the ferric salt and the sulphuric acid act upon the proteid-aldehyde compounds in some way, unknown, and form the violet colored substances. The work will be continued.

A COLLOIDAL COMPOUND OF STRYCHNIN AND ITS PHARMACOLOGY.[1]

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(Received for publication, February 26, 1906.)

During the course of some experiments on the effects of salts, alkaloids, etc., on catalysis and enzymotic action, the author in conjunction with Neilson used organ extracts and hydrogen peroxide mixed with the salts or alkaloids. In case a mixture of these three substances was allowed to stand for a day or two, it was observed that a precipitate usually resulted. At that time, which was several years ago, it had only recently been learned that a fixation of toxins by specific body tissues occurred, and this acted as an impetus to a revival of the old theory that animal tissues are capable of fixing alkaloidal poisons. It occurred to the author that it might be possible that this precipitate formed by the alkaloid, animal tissue, and hydrogen dioxide might be a definite chemical compound of the alkaloid and some constituent of the protoplasm.

Starting with a knowledge that a mixture of hydrogen dioxide and extracts of pancreas or kidney and a salt or alkaloid, produced a precipitate on standing, the idea was conceived of substituting for the organ extract, the white of egg. Three separate solutions were made as follows:

I	II	III
25 c.c. albumin.	25 c.c albumin.	25 c.c water.
25 c.c. hydrogen dioxide.	25 c.c. water.	25 c.c. hydrogen dioxide.
25 cc. $\frac{N}{100}$ strychnin citrate.	25 c.c. $\frac{N}{100}$ strychnin citrate.	25 c.c. $\frac{N}{100}$ strychnin citrate.

It was at first thought that the results were to be entirely

[1] A preliminary report of this investigation was made to the American Physiological Society, December, 1905. *Amer. Journ. of Physiol.*, xv, p. 22, 1906

negative, as nothing noteworthy happened in any one of the solutions during the first few days. It was observed, however, that there was in No. I a slight milky appearance which did not exist in Solution No. II. This gradually increased and became more perceptible. At the end of a month or six weeks, the reaction seemed to be complete; at least no further change was discernible. At this time the solution was usually of a milky grayish color, and was slightly opalescent. It had the consistence of a slightly fluid jell. In some cases the cylinder in which the substance was contained could be inverted without causing a dislodgment of the contents. In some of the experiments a quite different result was obtained, but this was seldom. In an occasional one a white flocculent precipitate resulted, instead of the jell. In one other there occurred a gelatinous precipitate of the same general appearance as the jell, but there was a clear liquid above it. Tests showed though that this clear liquid was of the same nature as the precipitate. When yolk of egg was substituted for the white, a similar change occurred in the mixture.

Solution No. II does not undergo a change in any way analogous to that noticed in Solution No. I. As there is no peroxide of hydrogen in that flask there usually results a bacterial decomposition in the course of a few days. Other antiseptics such as dilute hydrochloric acid and thymol have been substituted for the hydrogen dioxide. These prevented the putrefaction but in no case did the same change occur in the solutions in which these antiseptics were used as when the dioxide of hydrogen was used. The hydrochloric acid was used on the assumption that the acid of the peroxide might be responsible for the peculiar change observed. It was thought that the compound might be a simple acid albuminate, as it resembled an acid albumin somewhat. But such was found not to be the case.

The chemical tests were tried upon all three solutions. The results were negative except on No. III. But this was to be expected, since it is a well-known fact that organic matter in stomach contents must be removed before the chemical tests for strychnin are obtained. This fact undoubtedly explains the failure of Salant[1] to find strychnin in the colon of rabbits.

[1] *Journ. of Med. Res.*, xii, p. 41, 1904.

The physiological tests were then made. Two c.c. of each of the three mixtures were injected into the lymph spaces of different frogs of approximately the same size. The frogs receiving doses from Solutions Nos. II and III developed tetanus in about three minutes, and died in the course of ten to fifteen hours. The frogs injected with Solution No. I showed absolutely no effects for about three or four hours, at which time they showed the early strychnin symptoms, and at the end of four or five hours developed the typical strychnin tetanus. Death followed at the end of twenty to thirty hours or recovery took place gradually. The chances of recovery seemed better for those injected with Solution No. I than either II or III. It was observed in case of Solution No. I that the older the mixture was, the longer was the time required after injection for it to produce the strychnin effects.

Sufficient of Solution No. I to contain thirty times the lethal dose of free strychnin was then given to a rabbit subcutaneously. No effect was noticed, except that on the following day it seemed that the animal had some of the early symptoms of strychnin poisoning. But the strychnin tetanus did not develop. The same dose per kilo of body weight was then given to a rabbit per stomach. The rabbit developed marked strychnin tetanus in about three hours, and died in three hours and fifteen minutes after the administration of the substance.

The chemical behavior of the solution toward certain reagents was next ascertained. The jell is freely soluble in water, making a slightly milky, homogeneous, colloidal solution with a trace of opalescence. Heating the solution causes the milkiness and opalescence to disappear, and neither returns on cooling. Heat does not coagulate the solution. The reaction to phenolphthalein is not alkaline; to litmus it is very faintly alkaline. A small amount of any concentrated acid causes a precipitate which is soluble in an excess of the acid. Boric and other weak and slightly soluble acids have no effect upon it. The xanthoproteic reaction and Millon's test were obtained.

The following list of salts used in about $\frac{M}{1}$ or $\frac{M}{2}$ concentrations did not cause a precipitate: rubidium chloride; sodium chloride, bromide, sulphate, carbonate nitrate, and tartrate; lithium chloride; potassium hydrate, bromide, sulphate, cyanide, and ferrocyanide; calcium chloride and nitrate; strontium chloride; barium chloride; and magnesium chloride and sulphate. The following salts in $\frac{M}{5}$ concentrations or weaker produced a precipitation of the colloidal solution: cobalt chloride, nitrate and sulphate; manganese chloride; copper chloride, sulphate, and acetate; nickel chloride; uranium nitrate; silver nitrate; lead acetate; cadmium nitrate and sulphate; zinc acetate; ferric chloride; and aluminium sulphate. Iodine solution, chloroform, and ether do not precipitate the solution, but alcohol does.

The solutions in a number of cases from which the precipitate had been separated were then tested for strychnin by the ordinary chemical tests; but the results were negative. The colloidal mixture was digested with papain and also with trypsin. The clear filtrate was tested for

strychnin chemically, and the results were negative. This may have been due to the fact that some organic matter still remaining in the solution interfered with the tests; but it is also possible that the albuminate was not completely digested. The digested material was then injected in the usual amounts into frogs. Tetanus developed in the course of fifteen to twenty minutes, and the frogs died in the course of eight or ten hours. This slight retardation of the onset of the symptoms over that of Solution II or III may have been due to the dilution of the solution occasioned on the addition of the enzyme solution. Or it may have been due to the strychnin's still remaining in an easily separable form in combination with a portion of the albumin molecule.

DISCUSSION OF THE FIXATION OF STRYCHNIN BY ANIMAL TISSUES.

Widal and Nobecourt[1] mixed strychnin with the pulp of brain, liver, kidney, etc.; an amount of the mixture containing a fatal dose of strychnin was injected into a frog, and the drug produced no effects. These workers were of the opinion that some chemical reaction had occurred between the alkaloid and some constituent of the protoplasm. These results were corroborated by Thoinot and Brouardel,[2] who further showed that various other materials as potato, charcoal, chalk, etc., had the same effect when mixed with strychnin. They discovered also by chemical tests that the filtrate from these mixtures contained no strychnin. Hatcher[3] found that a much larger dose of strychnin could be borne by a frog if the strychnin was injected after being mixed with a solution of acacia.

The interpretation of the preceding results is not clear. Some physical or chemical factor is probably the basis of each. It is conceivable that the absorption of the drug is inhibited by the presence of the organic material; or that its liberation from a chemical union with the organic substance is so gradual that its maximum effect can not be obtained. The rate of absorption would undoubtedly also be slowed as a result of the organic material, even though there was a chemical union between it and the strychnin. The dilution of a solution of strychnin as shown by Meltzer,[4] is of considerable importance as regards the toxicity of a definite quantity of the drug. An amount of

[1] *La semaine médicale*, 1898, p. 93.
[2] *Ibid.*, 1898, p. 140.
[3] *Amer. Journ. of Pharmacy*, lxxiv, p. 283, 1902.
[4] *Journ. of Exper. Med.*, v, 1901.

strychnin which is fatal if injected when dissolved in a small amount of water produces little or no effect if administered when dissolved in a large quantity of water. Another factor which is of importance is that the elimination of strychnin begins very soon after its administration. This is true of a therapeutic dose, as has been shown by Adam,[1] Kratter,[2] Ipsen,[3] and others, and would probably be equally true with a toxic dose. It has not been stated by any of the previous workers on this subject whether any marked retardation of the onset of the strychnin effects occurred when it was administered in these organic mixtures.

Von Czyhlarz and Donath[4] devised an ingenious method, by which they obtained results which seemed to them to support the theory of the fixation of strychnin by animal tissues. The experiment consisted of ligating the hind leg of a guinea-pig, injecting into the tissues below the ligature a lethal dose of strychnin, and removing the ligature after a few hours. On the re-establishment of the circulation, a general distribution of the poison occurred. The characteristic effects of the strychnin did not develop. The explanation of this as offered by these investigators was that the alkaloid while being in contact with the tissues of the leg—muscle, blood, etc.,—was in some way neutralized *in vivo*. The natural assumption was that it was fixed by some constituent of the cells or fluid of the body as is the case with bacterial toxins.

Meltzer and Langmann[5] repeated the preceding experiments upon guinea-pigs and also performed the same experiment upon frogs and rabbits—animals which are more susceptible to the action of strychnin than the guinea-pig. The results of their experiments were positive upon the guinea-pig, but were negative upon the other animals with which they worked. It was suggested by Meltzer and Langmann that the experiments on the guinea-pigs were positive because of an impaired absorption, as a result of the inhibited circulation. (Just why an impaired

[1] *Beitr. z. ger. Chem.*, St. Petersburg, iii, 1872.
[2] Huseman's *Handbuch der Toxicologie*, Berlin, 1862, p. 510.
[3] *Wien. med. Wochenschr.*, 1882, p. 214.
[4] *Centralbl. f. inn. Med.*, 1900, No. 13.
[5] *Journ. of Med. Res.*, ix, p. 19, 1 03.

absorption exists in the one case and not in the other is not explained.)

Klein[1] repeated the experiments upon guinea-pigs and found that strychnin appeared in the urine within a very little while after its administration into the ligatured leg. Possibly then the proper explanation of von Czyhlarz and Donath's results was not that the strychnin was fixed by the tissues, but that a general and gradual elimination, and a slow absorption decreased the toxic effects of a dose. Carrara[2] repeated the experiments of von Czyhlarz and Donath on nephrectomized instead of normal animals. He argued that the greatest source of elimination was in this way excluded so that the slowness of absorption was unimportant and, consequently, that the positive results which he obtained supported the hypothesis regarding the fixation of strychnin by the tissues. Ehrlich[3] held firmly to the view that alkaloids, being foreign to animal organisms, are not capable of being fixed by the tissues; and he asserted only a few years ago that there is no adaptation or immunization for strychnin. But now an antitoxin has been produced. H. Meyer[4] injected subcutaneously into a rabbit very gradually large amounts of strychnin. He obtained the blood serum of the rabbit, and found that when it was injected with fatal doses of strychnin, the strychnin effects were prevented. The various tissues of the immunized animal had the same power as the blood serum. This artificial immunity lasts for three or four or more days. Just how much bearing these experiments on immunity might have on the explanation of von Czyhlarz and Donath's results is impossible to say. But it is quite possible that the guinea-pig may be capable of producing a degree of immunity against a dose of strychnin in a comparatively short time.

In the author's experiments, a mechanical inhibition of the absorption of the alkaloid by the albumin can scarcely account for the late appearance of the strychnin tetanus in Solution No. 1, since it contained the same amount of albumin as Solution

[1] *Zeitschr. f. Hyg.*, xxxvi, 1900.

[2] *Centralbl. f. inn. Med.*, 1901, No. 20.

[3] *Zeitschr. f. Heilk.*, Int. Med., 1901.

[4] *Berl. klin. Wochenschr.*, 1905, No. 38, p. 1225.

No. II. The dilution is in no way accountable as the concentration of the strychnin in each of the solutions was the same.

The author is inclined to believe that the mixture of hydrogen dioxide, albumin, and strychnin in some way chemically fixes the alkaloid; this probably is accomplished by the albumin. Just what part the dioxide of hydrogen plays is impossible to say. The experiments so far indicate that even though it may not enter into the compound, it seems to be essential for the reaction, as other antiseptics tried do not produce the same effects. It is probable that even if the peroxide enters into the chemical combination, not a very large amount is used, as considerable free oxygen is liberated. A flask containing the mixture will explode if tightly corked. In the case where the tissue extracts were used, the oxygen was set free very rapidly, and the precipitate or jell formed in a comparatively short time. But in the case where the albumin was used, the oxygen was liberated very slowly, and a correspondingly long time was required for the completion of the chemical reaction. It seems that the liberation of the oxygen is a factor upon which the reaction depends. This suggests the possibility that the strychnin may undergo oxidation. But even if this is so, it is evidently reduced back to its normal structure when taken into the body, for the typical strychnin tetanus never fails to appear after a few hours if the dose is sufficient. The mixture of hydrogen dioxide, water and strychnin (No. III) produces tetanus just as quickly as No. II, in which there is no hydrogen dioxide. This shows that it is very unlikely that the chemical change is one of oxidation.

Another experiment, which will be taken up in a later communication, adds support to the above hypothesis or explanation. Adrenalin chloride when mixed with hydrogen dioxide very rapidly becomes oxidized and takes on a pinkish coloration. But if albumin is first mixed with the adrenalin chloride the addition of the hydrogen dioxide does not produce any coloration.

Further support of the idea that in this jell there is a definite albuminate of strychnin are the experiments in which the mixture was administered to rabbits in thirty times the lethal dose of pure strychnin subcutaneously and by the stomach. In the former case where the absorption and the digestion of

the compound could go on but slowly, practically no effects of his enormous dose were obtained. But in the latter instance, when the compound was introduced into the stomach where active and plentiful amounts of proteid enzymes existed and hence where both digestion and absorption could take place fairly rapidly, tetanus was developed in three hours, and death rapidly ensued.

The conditions in the body are not so different from those in the test-tube containing albumin and hydrogen dioxide. in the former, active oxidative processes are occurring. In the latter, an active condition of the oxygen exists. If a chemical combination of the strychnin really occurs in the test-tube, it is quite reasonable to assume that the same may occur in the body. This may be the basis of immunity for strychnin. The tissues of an animal which is immune to strychnin would be capable of uniting quite rapidly with the strychnin, and thus remove it from its free state; it must be considered on this hypothesis that the typical strychnin symptoms result only when the drug is in a free state.

SUMMARY AND CONCLUSIONS.

1. A mixture of hydrogen dioxide, albumin, and strychnin will *undergo in the course of about six weeks' time*, a change,—probably a chemical change,—in which the previously thin, watery fluid is transformed into a jell, or throws down a white, flocculent precipitate.

2. Neither of the results occurs or is even indicated, if water is substituted for either the hydrogen dioxide or albumin.

3. Neither does the jell or the precipitate form if other antiseptic solutions, as hydrochloric acid or thymol, are substituted for the hydrogen peroxide.

4. If adrenalin chloride is substituted for the strychnin a jell is formed having the same appearance as is the case with strychnin.

5. The fact that the adrenalin chloride does not turn a pinkish color in the mixture with albumin and hydrogen dioxide as it does when mixed with hydrogen dioxide alone is significant that a chemical combination between the adrenalin and albumin is formed in the presence of hydrogen dioxide.

6. It seems that any salt or alkaloid and probably other chemical substances as well will form a jell or precipitate when mixed with albumin and dioxide of hydrogen.

7. Yolk of egg or organ extracts may be substituted for the albumin in this mixture with the formation of a similar jell.

8. The presence of organic material with strychnin, as found by other workers, is very likely to mask the chemical tests for it.

9. The administration of an amount of this jell which contains many times a lethal dose of strychnin produces tetanus only after four or five hours. The strychnin mixed with either albumin or hydrogen dioxide alone produces its effects in three to five minutes.

10. An amount of this jell containing thirty times a lethal dose of pure strychnin may be given a rabbit subcutaneously without producing toxic effects.

11. The same dose if administered per stomach produces death in a little over three hours.

12. For the reasons stated in the discussion it seems fair to conclude that the strychnin is not oxidized by the hydrogen dioxide.

13. It seems also that the conclusion is warranted that the strychnin of the mixture of hydrogen dioxide, albumin, and strychnin is in some way chemically fixed by the albumin so that its effects are not obtained when introduced into the body until after the alkaloid is separated from the albumin by digestion.

SOME REMARKS ON THE PROPOSITION THAT THUDICHUM'S PHRENOSIN AND THIERFELDER'S CEREBRON WERE IDENTICAL.

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(Received for publication, May 22, 1906.)

In a recent paper from this laboratory Posner and I expressed the conviction that protagon was a mechanical *mixture* of several substances in considerable proportions.[1] In the introduction to a description of our experiments we referred to some of the products that have been obtained from the protagon mixture and, in the course of our remarks, we incidentally expressed the opinion that Thierfelder's cerebron, Gamgee's pseudocerebrin, Koch's cerebrin, and certain other cerebrins (see page 165), were identical with Thudichum's phrenosin. Shortly after the publication of our paper, Thierfelder[2] objected to such a characterization of cerebron.

Although this difference of opinion regarding the identity of phrenosin and cerebron has no bearing whatever on the conclusion that was expressed by us regarding the chemical heterogeneity of protagon—a conclusion that confirmed a previous observation of Thierfelder's,—I desire nevertheless to refer briefly to Thierfelder's reasons for objecting to our deduction that phrenosin and cerebron were identical, and again to urge for Thudichum's work in neurochemistry the generously fair estimate I believe it deserves.

Thierfelder's rejoinder to our remarks was confined in the main to observations on the elementary composition and the decomposition products of phrenosin and cerebron. The following statements will also be directed to the same phases of the subject.

ON THE ELEMENTARY COMPOSITION OF PHRENOSIN (CEREBRON).

Among our reasons for concluding that "phrenosin and cerebron

[1] Posner and Gies, this Journal, i, p. 59, 1905. See also, Barbieri, *Compt. rend. de l'Acad. dessci.*, Paris, cxl, p. 1551, 1905.

[2] Thierfelder, *Zeitschr. f. physiol. Chem.*, xlvi, p. 518, 1905.

appear to have been identical"[1] was the striking similarity between the figures representing the elementary composition of phrenosin and those of cerebron, although the two products were separated by different methods and the analytic results for the elementary composition of each were obtained independently at periods separated by about 20 years.[2] Other reasons for the same conclusion will be stated later. The significance of this similarity in elementary composition was emphasized in our minds by the fact that it is difficult satisfactorily to separate and prepare fatty and fat-like substances individually, in uniformly pure condition, from tissue extracts.

Thierfelder[3] opened his discussion of the elementary composition of phrenosin with the following remark:

"Im Jahre 1874 wendet Thudichum die Bezeichnung Phrenosin zum erstenmal auf eine aus dem Gehirn isolierte phosphorfreie Substanz an, welche bei der Analyse als höchsten Kohlenstoffgehalt 66,6% gab und für die er verschiedene Formeln $C_{35}H_{69}NO_8$, $C_{34}H_{67}NO_8$, $C_{34}H_{69}NO_8$, berechnet. Später (*Journ. f. prakt. Chem.*, N. F., xxv, S. 521, 1882) erklärt er diesen Körper selbst für eine Mischung und verwirft die Formeln." (Page 518.)

With regard to Thudichum's *original* remarks about phrenosin in the *Reports of the Medical Officer of the Privy Council and Local Government Board* (1874), Thierfelder stated the following in a foot note (3) in connection with the above:

"'Researches on the Chemic. Constitution of the Brain.' Appendix Nr. 5 to *Reports of the Medical Officer of the Privy Council and Local Government Board.* New Series, Nr. III. Dieses Buch konnte ich weder auf der Kgl. noch auf der Universitätsbibliothek in Berlin und Göttingen erhalten. *Die obigen Angaben entnehme ich einer Arbeit von E. Drechsel* (Journ. f. prakt. Chem., N. F., Bd. XXV, S. 190, 1882), *in der die Untersuchung von Thudichum besprochen wird.*" (Page 518.) The inaccuracy of Drechsel's comment in this connection is referred to in the footnote on page 163.

I regret to say that the above quoted remarks about phreno-

[1] Posner and Gies, *loc. cit.*, p. 110.

[2] This similarity in composition suggested to us *isomerism* also, as was intimated in the following remark on page 73: "It seems singular that Thierfelder did not happen to discover, at the same time and on the same pages of Thudichum's book (1901) to which he referred, the fact that cerebron was either identical or isomeric with phrenosin."

[3] Thierfelder, *loc. cit.*

sin are not completely in harmony with the facts to which they allude and are therefore misleading. Unfortunately, Drechsel, from whom Thierfelder obtained his information on this phase of the subject, did not succeed in correctly reviewing all of Thudichum's data in this connection.[1]

[1]The volume containing Thudichum's original remarks on phrenosin (1874), which Thierfelder states he was unable to find, may be obtained at a low price and without difficulty. It was procured for me by Stechert & Co., of New York, a long time ago and was freely consulted by us in our protagon work in this laboratory. Thudichum frequently insisted that his work was unfavorably criticised by many who evidently did not take the trouble to read his papers. Such references to his work as those made by Maly (*Jahresbericht der Thier-Chemie*, v, p. 204, 1875), Drechsel (referred to in the quotation on p. 160 from Thierfelder's paper), and Parcus (*Journ. f. prakt. Chem.*, xxiv, p. 310, 1881), have undoubtedly served to misinform many and to prevent the study of Thudichum's papers that his work assuredly deserved. On the other hand, efforts to express truth without bias in this connection, which is evidenced admirably in such papers as those by Koch (*e. g., Amer. Journ. of Physiol.*, xi, p. 303, 1904), will do much to enforce the justice to Thudichum in this connection that was denied him before his death.

I fail to understand how students of neurochemistry can expect to maintain correct perspective in their work without making themselves familiar with Thudichum's investigations. His contribution of 1874, which Thierfelder states he never saw and which never was reviewed adequately in German, was the most important neurochemical publication up to that time, and it presents a view of brain chemistry which, for comprehensiveness of range and seriousness of attention to the work in hand, has seemingly not been surpassed by any other observer in a similar investigation. It has scarcely ever been referred to.

In his introductory remarks "To the Lords of Her Majesty's most Honorable Privy Council," in the report of 1874, the medical officer, John Simon, commented upon Thudichum's work as follows:

"I need hardly observe that a research of such interest—an endeavor so to unfold the very complex chemistry of the normal brain as to make the abnormal processes chemically intelligible, must, as regards the interest and importance of its object, rank highly among the exact studies by which physiology hopes to give light to medicine; but I would particularly note that while certainly it is among the most interesting and important of such endeavors, so it certainly must be counted among the most difficult; and I therefore refer with particular satisfaction to the very remarkable evidences of success which Dr. Thudichum is already able to show."

About a year ago, I gave all my spare time for several months, in connection with our work on protagon, to a critical examination of the

Analyses of two comparatively pure preparations of "phrenosin" were reported in Thudicum's first publication (1874), on pages 184 and 187, as follows, of which that from ox-brain was considered the purer:

Human Brain.			*Ox-brain.*		
Percentage results of analysis	Calculated no. of atoms in the molecule ("÷by N=1")	Empirical formula adopted	Percentage results of analysis	Calculated no. of atoms in the molecule ("÷by N=1")	Empirical formula adopted
C 66.1	35.1	$C_{35}H_{69}NO_8$	66.6	33.84	$C_{34}H_{67}NO_8$
H 11.0	70.6		11.2	68.29	
N 2.2	1.0		2.3	1.00	
O 20.7	8.1		19.9	7.62	

The *third* formula mentioned by Thierfelder and given by Thudichum, p. 185 ($C_{34}H_{69}NO_8$), was derived by *calculation* of the *probable* phrenosin content of some *mixed* products that were designated in each case "*cerebrin and phrenosin.*" The formula $C_{34}H_{67}NO_8$ was the one that Thudichum derived from his analysis of what he called "*purest* phrenosin."[1] Consequently, instead of his three *formulas* indicating inaccuracy or uncertainty, they reflected the then prevalent custom of putting analytic results for individual preparations in formular arrangement. The average analytic data correspond fairly well with the *one* formula that Thudichum *provisionally* assigned in 1874 to phrenosin, on the basis of his results with the "*purest*" phrenosin prepared up to that time.

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extended *historical* review appended to Thudichum's experimental records in the report of 1874 and was impressed by the fact that Thudichum's compilation was remarkably complete and free from error. His conclusions indicated that he had mastered the entire subject and had carried into his work a liberal view and a broad-minded purpose. The bitterness of the personal allusions which marred many of Thudichum's subsequent publications appears to have been engendered by the unappreciative reception and unfair treatment his observations were accorded by some of his most influential colleagues. It should not be permitted to blind us, however, to the merits of his work that are discernible in abundance among his mistakes.

[1] This product was not considered by Thudichum to be entirely free from impurity.

That Thudichum did not regard these observations as final must be inferred from the following remarks of his in 1874:

"It is thus shown that different human brains yield immediate principles of the cerebrine series, which apparently greatly differ in elementary composition, though they are very similar in chemical reaction; but, by a simple *hypothesis*, they are reducible to two *primary forms*, *mixed* in varying proportions:

"Monoamidated form— $C_{34}H_{67}NO_8$-phrenosine.

"Diamidated form — $C_{34}H_{68}N_2O_8$-cerebrine." (Page 186.)

"While then there may be some degree of *uncertainty* as to the absolute purity of some of the principles evolved, there can be none as to their *striking individuality;* and, as regards this, the positive evidence of their peculiar and distinctive qualities is so strong that the fact of their not uniformly answering to certain other criteria is, in my opinion, quite insignificant." (Page 203.)

"The distinction of individuals is more difficult in the cerebrine group, the members of which are more similar to each other in the properties as yet known. Of the individuality of kerasine there can, I think, be no doubt; at least if strictly uniform crystallizability be of any value as a criterion. *More doubt may be allowed in the matter of phrenosine*, where these doubts have led me to a great number of experiments to establish the purity and fix the size of the molecule. As regards the rest of the group the information given is little more than a preliminary pioneering account, valid as far as it goes, but not in any particular of a final nature." (Page 203.)

The subsequent revision (1880) that Thudichum made of his opinion regarding the *molecular* character of phrenosin (to which revision Thierfelder makes inaccurate allusion) was fully foreshadowed by the doubts frankly expressed in 1874. It seems to me, in the light of subsequent events, that Thudichum's original views do credit to his judgment in estimating correctly the value of his preliminary data.[1] Thierfelder himself has made a similar revision of his figures for cerebron, as a result of further analysis.

[1]In the paper by Drechsel, from which Thierfelder has drawn his information in this connection, there are a number of sarcastic references to the remarkable qualities of phrenosin—a substance with more than one formula! The above mentioned considerations among others seem to have been ignored by Drechsel. Drechsel's derisive remarks were applied also to kerasin. The observations by Kossel and Freytag (*Zeitschr. f. physiol. Chem.*, xvii, pp. 439–445, 1893) were in effect early confirmations of Thudichum's work in that connection, as opposed to Parcus's opinions relating to his own work done under Drechsel's direction.

The two quotations (page 160) from Thierfelder's remarks have no bearing, therefore, on the phrenosin *subsequently described* by Thudichum (in 1880 and frequently since) and to which Posner and I referred.

A few years after the publication of the preliminary observations on phrenosin Thudichum made a more detailed study of it.[1] He recognized the fact that the *best* of his later products contained impurities (kerasin, or phosphatid, or both), which did not amount, however, to more than 1 per cent. The average data of his determinations of percentage elementary composition of phrenosin that were published at that time (1880 and since) were the following:

C	H	N
67.957	11.426	1.997

From a study of the qualities of its decomposition products, and with due regard to the presence of recognized impurity, Thudichum finally assigned to phrenosin itself the formula $C_{41}H_{79}NO_8$, which represented the following *hypothetical* composition (see page 172):

C	H	N	O
69.00	11.08	1.96	17.96

Thierfelder's average results of analysis for percentage elementary composition of cerebron, as *revised* to date, are the following[2]:

C	H	N
69.19	11.35	1.76

In harmony, however, with the course pursued by Thudichum, Thierfelder has concluded, with the help of his results on the de-

[1] Thudichum, *Reports of the Medical Officer of the Privy Council and Local Government Board*, pp. 143–206, 1880; also, *Annals of Chemical Medicine*, ii, pp 1–53, 1881, and *Journal für praktische Chemie*, xxv, pp. 19–28, 1882. The latter paper merely summarizes briefly the essential results pertaining to phrenosin. Numerous reprints appeared subsequently. The latest may be found in Thudichum's last volume, *Die Chemische Konstitution des Gehirns des Menschen und der Tiere*, pp. 178–214, 1901.

[2] As given in his latest publication on the subject (*loc. cit.*, p. 520, 1905). The earlier figures were C, 69.16; H, 11.54; N, 1.76. Nitrogen was not determined in the later preparations, otherwise its figures might have required revision also.

composition products of cerebron, that the formula for cerebron is $C_{48}H_{93}NO_9$, which formula corresponds to the following *hypothetical* composition, as calculated by Thierfelder:

C	H	N	O
69.65	11.24	1.70	17.41

The harmony between the analytic results and the *calculated* data, for cerebron, is closer than that between the similar figures for phrenosin, but the agreement is not perfect. The disagreement is sufficient to justify the conclusion that the cerebron preparations, like those of phrenosin, contained small proportions of impurities, or else, as was also the case with Thudichum's phrenosin, the analytic results were not perfect. Can not both be said of all analyzed samples of every substance? Can any analysis yield perfect results? Until *ideal* conditions of preparation and analysis are attainable it is obvious that formulas can represent at best only very probable truth as deduced from somewhat *imperfect* data. In his observations on the slight amount of impurity in Thudichum's product, Thierfelder seems to ignore these obvious facts. He also seems to be oblivious to their bearing on his own. (See page 179 of this paper.)

In our paper, Posner and I presented the following table (p. 74):

	C	H	N	O
Thudichum's phrenosin (1874[1])	69.00	11.08	1.96	17.96
Gamgee's pseudocerebrin (1880)......	68.89	11.87	1.83	17.41
Wörner and Thierfelder's cerebron (1900)	69.16	11.54	1.76	17.54
Parcus's cerebrin (1881).............	69.08	11.47	2.13	17.32
Kossel and Freytag's cerebrin (1893)..	68.99	11.52	2.25	17.24
Koch's cerebrin (1902)...............	68.73	11.83	1.64	17.80

Thierfelder asserts that this table will awaken misconceptions of the facts to which it relates. He objects to our comparison of his *analytic* averages for the elementary composition of cerebron, with similar data corresponding to the formula that was *calculated* by Thudichum for phrenosin. Thierfelder suggests that we should have given, instead of the figures corresponding to what Thudichum regarded as ideally *pure* phrenosin, the related figures that Thudichum obtained for what he clearly recognized

[1]The numerals in parenthesis represent the year in which each substance was first described.

as slightly *impure* phrenosin. The two sets of figures are brought together below, to which are also appended for easy comparison the similar data for cerebron:

Phrenosin	C	H	N	O
Thudichum's average percentage *analytic* data for elementary composition..............	67.96	11.43	1.99	—
Thudichum's *calculated* percentage data corresponding to his formula, $C_{41}H_{79}NO_8$........	69.00	11.08	1.96	17.96
Cerebron				
Thierfelder's average percentage *analytic* data for elementary composition..............	69.19	11.35	1.76	—
Thierfelder's *calculated* percentage data corresponding to his formula, $C_{48}H_{93}NO_9$........	69.65	11.24	1.70	17 41

The differences in this connection, to which Thierfelder, in his reply to our observations, attaches so much importance, are suggestive of the difference between tweedledum and tweedledee. This is particularly true on account of the very great difficulties connected with accurate determinations of carbon and hydrogen in lipoid products, in which the proportionate contents of these elements are relatively large, and because different methods of analysis were employed; especially, also, in view of the differences between Thierfelder's own sets of figures, although Thierfelder attributes no significance to them. We deliberately gave the preference in our table to the figures which the author himself (Thudichum) considered the most accurate expression of the truth as he conceived it.[1] The same figures are generally given for phrenosin, so that we have done nothing unusual. See Koch's[2] tabulation, for example.

It is noticeable that Thierfelder does not object, however, to our inclusion (in the same table) of Gamgee's figures for pseudocerebrin, evidently because Thierfelder himself previously stated that pseudocerebrin and cerebron were *identical*—and that, too, regardless of the fact that Gamgee's analytic results for elementary composition of pseudocerebrin vary from Thierfelder's for cerebron almost as much as do Thudichum's for phrenosin, and

[1] By an oversight we failed to do the same for Thierfelder, but in the case of cerebron, also, the difference between the two sets of values is of no practical importance, as the figures above indicate.

[2] Koch, *Amer. Journ. of Physiol.*, xi, p. 310, 1904.

despite the further fact that the formula calculated by Gamgee for pseudocerebrin was $C_{44}H_{92}NO_8$, whereas the formula Thierfelder assigned to cerebron was $C_{48}H_{93}NO_9$. Why then the acceptance by Thierfelder of one set of data as equivalent to his own and not the other?

The only apparent reason for Thierfelder's entire acceptance of Gamgee's results as fully indicative of the identity of pseudocerebrin with cerebron, in spite of the differences alluded to, is given in the following quotation from his paper two summers ago, in which acknowledgment was made of Gamgee's suggestion that the two were the same (*Zeitschr. f. physiol. Chem.*, xliii, p. 22, 1904):

"Herr Gamgee fügte hinzu, dass er bei der Durchsicht seines Laboratoriumjournals Zeichnungen des mikroskopischen Bildes des Pseudocerebrins, welche mit den von uns veröffentlichten völlig übereinstimmen, gefunden habe, und ferner unter dem 11. Juli 1879 die Eintragung, dass das Pseudocerebrin beim Erhitzen in der Kapillare bei 197° sich leicht orange färbt und bei 210° schmilzt.

"Wir hatten für das Cerebron im Mittel 69,16% C; 11,54% H; und 1,76% N gefunden (see table p. 165) und den Schmelzpunkt bei langsamem Erhitzen bei 209°, bei schnellem Erhitzen bei 212°.

"Darnach kann es nicht zweifelhaft sein, dass Pseudocerebrin und Cerebron identische Körper sind und dass die Priorität der Entdeckung A. Gamgee zukommt."

Although I agree perfectly with Thierfelder in his view that pseudocerebrin and cerebron were practically the same, it seems to me that their agreement in crystalline appearance and melting point does not offer as much assurance of the identity of cerebron with Gamgee's product as the many points of agreement between the physical and chemical properties of phrenosin and cerebron are in favor of its identity with Thudichum's. Thudichum does not appear to have made any observations on the melting point of phrenosin. Gamgee did not report a study of the cleavage products of pseudocerebrin.

The surprising thing about Thierfelder's ready acceptance of Gamgee's suggestion that pseudocerebrin and cerebron were identical, *in view of Thierfelder's emphasis upon the presence of slight proportions of impurities in Thudichum's product and the consequent rejection of phrenosin as equivalent to cerebron*,[1] is the

[1] See page 166 of this paper.

fact that Thierfelder completely ignored some of the following very definite statements made by Gamgee about pseudocerebrin,[1] although the sentence below in italics was among those quoted by Thierfelder himself in his second communication on cerebron[2] as a part of the statement there made *to show that pseudocerebrin and cerebron were completely and perfectly the same:*

"In addition to protagon and other phosphorized matters, there is always extracted from brain by alcohol at 45° C. a very considerable quantity of a body which, in order to distinguish it, the author *provisionally* termed *pseudo-cerebrin.* This body is less soluble in 80 per cent. alcohol at 45° C. than protagon, so that on subjecting impure protagon to repeated crystallization from 80 per cent. alcohol there accumulated residues consisting of the cerebrin-like body. The latter is a white, pulverulent body, very unlike protagon to the naked eye and separating under the microscope in the form of very large nodular masses. *After repeated recrystallization from alcohol it was found to be practically free from phosphorus (containing only 0.08 per cent.)*[3] . . . Whilst the author would refrain from speaking with confidence of the absolute purity of 'pseudo-cerebrin,' he has, however, no hesitation in asserting that Geoghegan's substance, from the method of preparation, is necessarily a mixture of the so-called pseudo-cerebrin just referred to with a 'cerebrin' obtained by the action of barium hydrate on protagon—and therefore *much more impure* than the body now *provisionally* described by the term of pseudo-cerebrin.

"Analyses of 'pseudo-cerebrin' found by the author to accompany protagon:

	(1)	(2)	(3)	(4)	Mean.
C............	68.97	68.95	69.01	68.67	68.89
H............	11.70	11.17	11.60	12.10	11.87
N............	1.76	1.95	1.64	2.01	1.83
O............	——	——	——	——	17.41
					100.00

"From the above analyses the author deduces for 'pseudo-cerebrin' the empirical formula $C_{43}H_{92}NO_8$."

Gamgee published no further observations on pseudocerebrin that I am aware of, except those communicated to Thierfelder and quoted on page 167. It is obvious that the pseudocerebrin described by Gamgee was quite as impure as Thudichum's phrenosin,

[1] Gamgee, *A Text-Book of the Physiological Chemistry of the Animal Body*, i, p. 441, 1880.

[2] Thierfelder, *Zeitschr. f. physiol. Chem.*, xliii, p. 21, 1904.

[3] See Thudichum's quoted remarks on page 174 of this paper.

and no more individualistic chemically. Yet Thierfelder denied the equivalence of phrenosin and cerebron because phrenosin contained a trace of impurity but accepted pseudocerebrin as identical with cerebron in spite of a similar content of impurity.

Thierfelder based his acceptance of pseudocerebrin as a perfect equivalent of cerebron on a number of points of resemblance, which, as I stated before, do not appear to be as important as more significant resemblances between phrenosin and cerebron, which Thierfelder ignored. Thus, he emphasized sameness of crystalline character, yet Gamgee said pseudocerebrin separated "in the form of very large *nodular* masses," *i. e.*, as seen under the microscope. Does the formation of *nodular* masses amount to much in this connection as an assurance of chemical sameness? Do not many *dissimilar* substances form such masses under similar conditions?

Thierfelder does not explain, in his rejoinder to our remarks, how it happens that Gamgee's avowedly *impure* pseudocerebrin and his own *pure*[1] cerebron had the same melting point. Do the statements by Gamgee (page 168), show that agreement in melting point is always a satisfactory sign of perfect chemical sameness, as Thierfelder would seem to have us infer is the case? See, in this connection, the foot note on page 173 of this paper.

Thierfelder made the following remark about Thudichum's results for phrenosin:

> "Die Einheitlichkeit des Phrenosins ist durch diese Analysen um so weniger bewiesen, als sie sich offenbar alle auf ein und dasselbe Präparat beziehen." (Page 519.)

But the same remark applies, apparently, to Gamgee's results, also. Why should the line be drawn at Thudichum's? (See page 170).

Thierfelder gave the table of analytic results for phrenosin, presumably to emphasize the variations in Thudichum's data. But it happens that Thudichum's results vary among themselves very little if any more than the foregoing results of Gamgee's that appear to be entirely satisfactory to Thierfelder. The table on the next page shows the point just mentioned, and also gives comparative data from Thierfelder's own records for cerebron:

[1] I say "pure" because Thierfelder has not recognized any impurity.

	Thudichum's analytic data for phrenosin quoted disapprovingly by Thierfelder (1906).			Gamgee's analytic data for pseudocerebrin quoted approvingly by Thierfelder (1904).			Thierfelder's analytic data for cerebron published by Wörner and Thierfelder[1] (1900).		
	Lowest	Highest	Maximum difference	Lowest	Highest	Maximum difference	Lowest	Highest	Maximum difference
C	67.67	68.56	0.89	68.67	69.01	0.34	68.75	69.46	0.71
H	11.23	11.62	0.39	11.17	12.10	0.93	11.13	11.82	0.69
N	1.69	2.34	0.65	1.64	2.01	0.37	1.58	1.96	0.38

I think I may be pardoned for raising an inquiry at this point as to just what it was, in Thierfelder's understanding of the matter, that Gamgee named, and referred to as, pseudocerebrin. Does Thierfelder accept, as pseudocerebrin, the *impure product*, with Gamgee's very precise statement before him regarding the percentage proportion of phosphorus in it and the implied content of phosphatid (as in the case of Thudichum's phrenosin products), or does Thierfelder mean to have us infer that in his estimation pseudocerebrin was *not the product itself* that was subjected to analysis, but the hypothetical substance with which the impurity was associated and which Gamgee recognized in his product *in spite of* that evident admixture of impurity? The first alternative is not the one that Thierfelder appears to have had in mind when the remarks of Gamgee that are repeated above

[1] Almost all of Thierfelder's data were individual results for eight *different* preparations, but even in spite of that fact, he will hardly object to an application to his figures, obtained by the latest and best methods, of standards of estimation he has himself applied to Thudichum's. Thierfelder does not recognize more than one kind of cerebron. Consequently, if his products were *perfectly pure*, as he seems to take for granted they were, how does he explain the variations in his analytic results? If his products were *perfectly pure* what does the fact that the results were obtained from "eight different preparations" have to do with the matter? His latest results vary within the extremes given above. If, however, the variations in his figures were due merely to unavoidable errors of analysis, how can Thierfelder be any more certain, on the basis of such defective results, of the *molecular* characters of cerebron, than Thudichum was of those of phrenosin on a similar basis, the sufficiency of which in Thudichum's case, however, Thierfelder denies? I am merely suggesting here that Thierfelder's criticism of Thudichum's results should be applied to his own also. I have already made it clear that I consider Thierfelder's criticisms as inept when applied to Thudichum's results as they would be if applied to Thierfelder's.

were quoted by him. But, if Thierfelder took the second alternative and, in so doing, granted to Gamgee (as every one would, of course,) the ordinary chemical insight necessary for him to make his conclusion a reliable one (and thereby accepted Gamgee's results of *deduction* from *imperfect* chemical data instead of holding him rigidly to his direct analytic results), how can Thierfelder object to granting that others may make equally satisfactory inferences under similar conditions in other connections? Has not Thierfelder himself come to the conclusion that cerebron is a substance with a composition different from that defined by the results of his analysis of it? What remains, therefore, of Thierfelder's objection that phrenosin and cerebron could not have been identical because of the slight amount of impurity in his phrenosin products that Thudichum recognized as frankly, and apparently estimated as accurately, as Gamgee did the impurity in the product composed almost wholly of the *hypothetical* pseudocerebrin? Has any cerebron product been perfectly, ideally pure? Could cerebron be thus obtained by any one? It is quite likely that if Thudichum and Gamgee had made their determinations of elementary composition under the favorable circumstances that conditioned Thierfelder's work, the three sets of figures would be in even more perfect accord than they are.

ON THE DECOMPOSITION PRODUCTS OF PHRENOSIN (CEREBRON).

The leading cleavage products of phrenosin were originally stated to be cerebrose, sphingosin, and neurostearic acid. Cerebrose was subsequently found to be identical with galactose.[1] Cerebron yields the same cleavage products, an observation that points to the main evidence of the chemical identity of phrenosin and cerebron. Similar facts pertaining to pseudocerebrin are lacking.

[1] In his latest volume (1901, p. 313) Thudichum offers the following definitions:

"*Cerebrose*, erster Name für den aus Phrenosin durch Chemolyse erhaltenen Zucker, $C_6H_{12}O_6$, *Galactose*, als Cerebro-Galactose zu kennzeichnen. Krystallisierte und amorphe Form.

"*Cerebroside*, Name für die Cerebro-Galactoside, deren Typus durch Phrenosin vorgestellt wird. Reihe von neutralen Edukten aus Hirn, die neben dem Zucker eine Fettsäure und ein stickstoffhaltiges Radikal bergen."

In a footnote on page 74 of our paper[1] the following statement appears:

"Thierfelder indicated the quantitative relationships of the radicals in cerebron (phrenosin) with the following equation (1905, p. 370):

$C_{48}H_{93}NO_9$	+	$2H_2O$	=	$C_{25}H_{50}O_3$	+	$C_{17}H_{35}NO_2$	+	$C_6H_{12}O_6$
Cerebron (phrenosin)				Cerebronic acid (neurostearic acid)		Sphingosin		Galactose

"On page 209 of the publication of Thudichum's (1901) to which Thierfelder referred, the same facts are shown by the following equation, which also appeared in various publications of Thudichum's more than twenty years ago:

$C_{41}H_{79}NO_8$	+	$2H_2O$	=	$C_{18}H_{36}O_3$	+	$C_{17}H_{35}NO_2$	+	$C_6H_{12}O_6$
Phrenosin (cerebron)				Neuro-stearic acid (cerebronic acid)		Sphingosin		Galactose

The trivial differences between the figures for elementary composition of phrenosin and cerebron account for the seemingly great differences between the formulas of the original substance that are given above. That Thierfelder's results were more nearly correct may be taken for granted. That the substances were the same seems evident."

The last three sentences in the above quotation make evident our conviction that neurostearic acid and cerebronic acid were essentially the same, in spite of the decided differences between the formulas. That the two products were practically identical seems to be indicated by the following figures, which represent the average percentage results of direct elementary analysis:[2]

	C	H
Neurostearic acid	75.94	12.64
Cerebronic acid	75.33	12.50

[1] Posner and Gies, *loc. cit.*

[2] Disregarding the slight proportions of impurities in each, Thudichum's phrenosin and Kossel and Freytag's cerebrin seem to have been identical products, as is indicated in the table on page 165. Kossel and Freytag found that on oxidation with *nitric* acid, phrenosin (cerebrin) yielded 68 per cent. (68.12%, 68.38%, 67.67%) of stearic acid. They commented on their acid product as follows. (*Zeitschr. f. physiol. Chem.*, xvii, p. 449, 1893):

"Die Schmelzpunkte der erhaltenen Fettsäuren lagen alle zwischen 70° und 71,5°. Die geringe Erhöhung des Schmelzpunktes ist wahrscheinlich darauf zurückzuführen, dass dieselben eine geringe Menge unzersetztes Cerebrin einschlossen. Durch fractionirte Krystallisation

These figures, which were obtained by two observers independently at periods separated by about twenty-five years, are in remarkably close agreement. It was this observation which stimulated our investigation of the phrenosin-cerebron matter. It was Thierfelder's failure to refer to this agreement that occasioned some of the remarks in our former paper to which Thierfelder has objected.[1] It seems to me that this striking agreement deserved some notice to say the least.

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wurden drei Proben dargestellt. Die beiden ersten Fractionen begannen bei 69° zu schmelzen und waren bei 70,5° völlig flüssig, die dritte Fraction begann bei 75° zu schmelzen und war erst bei 78° ganz geschmolzen. Demnach sind die erhaltenen Procentzahlen für die Stearinsäure um ein Geringes zu hoch. Dass in der untersuchten Säure wirklich Stearinsäure vorlag, wurde noch durch die Ueberführung in das Barytsalz und Analyse desselben nachgewiesen. Dasselbe enthielt 19,23% Ba; stearinsäurer Baryt verlangt 19,48% Ba, palmitinsäurer Baryt 21,17% Ba."

Thudichum assumed that neurostearic acid (which he obtained by oxidation with *nitric* acid and also by decomposition at 130° with 2% *sulfuric* acid) was an isomer of ordinary stearic acid. He recognized the probable presence of slight proportions of impurities.

Thudichum's neurostearic acid (crystallized from ether) melted at about 84° C, Kossel and Freytag's stearic acid (after filtering from nitric acid, washing with water and drying) at about 71° C and Thierfelder's cerebronic acid (crystallized from alcohol) at about 99° C. Thudichum found, however, that the curdy mass that was precipitated from a solution of a soluble salt of neurostearic acid by mineral acid did not melt at 95° C. The acid softened but did not become oily when floated on boiling water. The effect of a higher temperature in this connection was not mentioned.

Thierfelder appears to lay great stress on the difference between the melting points given by him and Thudichum for cerebronic acid (99° C) and neurostearic acid (84° C). Thudichum's observation on the acid-precipitated product is not taken into account by Thierfelder. The great difficulties in the way of accurate determination of the melting points of fatty products should not be ignored in this connection. The following quotation from Thierfelder's last revision of Hoppe-Seyler's *Handbuch der Physiologisch- und Pathologisch-Chemischen Analysen*, p. 86, 1903, expresses forcibly the universal experience in this connection with fatty and fat-like substances:

"Der eigentliche Schmelzpunkt (Stearin) ist 71,5°. Es ist aus thierischen Fetten kaum rein zu erhalten. Das unreine Stearin ist bei 53°–66° schmelzbar."

[1] We stated the following on pages 73-74:

"In his latest contribution on this subject, Thierfelder referred to the results of a decomposition of cerebron by an improved method—heating

In his remarks about the phrenosin that he used for his observations on the cleavage products, Thudichum wrote as follows (p. 182, 1901):

"Das Phrenosin wird in Alkohol gelöst, vom Schwefelcadmium abfiltriert und abermals krystallisiert. Zuletzt kommt ein Punkt, wo keiner der oben beschriebenen Prozesse, wie oft man ihn auch wiederholen mag, die Zusammensetzung oder das Aussehen des Phrenosins ändert. Dann enthält es noch meist von 0.02 bis 0.05%P, also ein halb bis ein Prozent Phosphatid. Dies muss die direkte Elementaranalyse stark beeinflussen, hat aber auf die durch Chemolyse zu erreichenden Produkte wenig Einfluss, so dass es nicht hindert, mit beiden Methoden zu einer ganauen Kenntnis der elementaren Zusammensetzung des Phrenosins zu gelangen."

The *recognized* impurity was, no doubt, missing[1] from Thierfelder's product. The impurity referred to may have raised the figure for carbon content of neurostearic acid. At the same time the two sets of figures for elementary composition agree as well as could be expected for a product with such a high carbon content and which was analyzed by two different observers at widely separated periods, after preparation by different methods. These facts make the agreement all the more striking and emphasize our deduction that the two substances were identical.

The differences between the percentage values of the formulas and the results of direct analysis may be seen below (Posner and Gies, *loc. cit.*, p. 73):

	Data of direct analysis[2] C	Data of direct analysis[2] H	Formula given	Corresponding composition required by the formula C	Corresponding composition required by the formula H
Thudichum's neurostearic acid (1878):					
The acid obtained from its ethyl ester	75.94	12.64	$C_{18}H_{36}O_2$	76.06	12.68
The crystalline acid	75.88	12.85			
Thierfelder's cerebronic acid (1904)	75.33	12.50	$C_{25}H_{50}O_3$	75.38	12.56

with methyl alcohol containing 10 per cent. of sulfuric acid. The proportions of the above-named decomposition products were determined. Again there was no reference to the obvious identity of cerebron with phrenosin nor to the significant resemblance between neurostearic acid and cerebronic acid. We believe the latter was merely a purer form of Thudichum's product. Thierfelder also refrained from making further direct reference to his confirmation of the essentials of Thudichum's original observations on sphingosin."

[1] If it was not entirely missing, probably only traces were present. I referred to this matter on page 165.

[2] In the previous paper, I gave by mistake, after neurostearic acid,

It seems probable that Thudichum was wrong in preferring the formula he selected for neurostearic acid. He failed to take into account in this connection the probable admixture of portions of the impurity that he knew was present in the phrenosin to begin with.[1] At the same time cerebronic acid is not yet so well defined in character that its composition can be regarded as settled beyond all doubt. The two formulas on page 174 are practically interchangeable. Each is *empirically* an approximate multiple of $C_8H_{17}O$.

Phrenosin and cerebron are admittedly the same in yielding galactose and sphingosin. Each also yields an organic acid of relatively high carbon content. Thudichum called this acid neurostearic acid, Thierfelder called it cerebronic acid. I am convinced that each was dealing with essentially the same product. I believe also, that Thierfelder's product was the purer of the two. It seems to me that phrenosinic acid would be the most satisfactory name for it.

Near the close of his last paper, Thierfelder laid great stress on the differences between the figures for composition and melting points of neurostearic acid and cerebronic acid, discrepancies that Posner and I assumed, quite correctly I think, were due to the presence of slight proportions of impurities in the products. I have already referred in detail to these differences and their minor import. (p. 172). In this connection Thierfelder wrote as follows:

"Es gibt nur zwei Möglichkeiten: Entweder hat Thudichum eine reine Säure in Händen gehabt und analysiert, dann sind Phrenosin und Cerebron verschiedene Körper. Oder er hat unreines Material untersucht, dann sind seine Angaben über die Zusammensetzung der Säure und damit auch des Phrenosins unrichtig und das Phrenosin wäre vielleicht als unreines Cerebron zu bezeichnen, aber nicht das Cerebron als Phrenosin.

"Eine sichere Entscheidung ist nicht zu treffen. Berücksichtigt man aber die zu Phrenosin und Cerebron führenden Darstellungsverfahren, die für beide Substanzen angegebenen Eigenschaften *und vor allem den*

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the higher figures (C, 76.69; H, 12.95) for the ethyl ester. The error was not at all favorable to the conclusion I was endeavoring to make evident. In the table on page 174 is given not only the figures *usually* presented for results of direct analysis of neurostearic acid, but also additional data of the same kind for the crystalline product (Thudichum, p. 210, 1901).

[1] See remarks in the footnote on page 173 of this paper.

für Thudichum's Arbeiten charakteristischen Mangel an Exaktheit, so wird man sich mehr der letzteren Auffassung zuwenden."[1] (Page 521).

I agree in *assuming*, with Thierfelder, that neurostearic acid was not quite as pure as cerebronic acid, but, instead of presuming that Thudichum's statements are materially in error regarding the *composition* of neurostearic acid, it is necessary to admit only that Thudichum's selection of his formula for neurostearic acid was mistaken: So was Gamgee's for pseudocerebrin, but that fact did not prevent Thierfelder from accepting pseudocerebrin as equivalent to cerebron (page 167). I also agree with the substance of the deduction indicated in the last sentence of the first of the two paragraphs just quoted, but I believe the same truth may be stated with greater regard for historical sequence by saying that cerebron is a purer form of phrenosin and that, disregarding the slight proportions of impurities *in both products*, each observer (Thudichum and Thierfelder) was dealing with the same substance.

It is proper to insist at this point that Thudichum gave the name phrenosin *not to the product that he analyzed but to the* substance that composed nearly all of that product and which a small amount of associated impurity did not hide from him. I have already drawn attention to the fact that Thierfelder has in effect done the same for cerebron. In discussing phrenosin, Thierfelder insists on recognizing only the practical data pertaining to a product containing a small amount of acknowledged impurity. More privileged treatment is accorded to pseudocerebrin (page 168). Who ever analyzed a mass of any substance that was not impure and who ever erected a formula that accorded perfectly with analytic data? To what do the names of substances refer? Are they given to compounds actually analyzed in the absence of all traces of others—to ideally pure substances, or do the names refer to compounds in *products*, in which the essential, fundamental, predominating substance, as it is conceived to be, is recognized? Sometimes impurities are discerned and duly accounted, sometimes they are not. In the case of phrenosin, impurity was recognized, in the case of cerebron, impurity does not seem to be conceded. Shall a different name be given to a substance every

[1]Italics are mine.

time a trifling amount of impurity is discovered in products made by methods that were presumed to yield that particular substance and nothing else? When slight proportions of impurities are recognized in products thus obtained, is it not quite satisfactory and customary to retain for the substance with which the impurity was associated, the name that was originally applied to such masses containing both? Is it any more desirable to give several names to the essential substance common to slightly different products obtained by *dissimilar* methods? Why drop the name phrenosin for cerebron in view of the facts in this connection?

REMARKS ON METHODS OF PREPARATION, PHYSICAL PROPERTIES, ETC., OF PHRENOSIN (CEREBRON).

With regard to the statement in the second paragraph of the remarks just quoted from Thierfelder's paper (p. 175), I might add that it has not been shown that Thudichum's methods of preparation were not as satisfactory as Thierfelder's. Thierfelder's objection to high temperature reminds me of the great precautions that used to be taken in this connection to prepare protagon, until Cramer[1] found that the same material could be obtained by extraction at the *boiling* temperature of 85 per cent. alcohol. Whatever differences have been discovered between the properties of phrenosin and cerebron, to which Thierfelder alludes indefinitely (Thierfelder does not say what they may be), are certainly buried under the resemblances. Some of the remarks made in the description of phrenosin are duplicated in the subsequent description of cerebron.

Thus, each is described as a snow-white substance, readily soluble in warm absolute alcohol and obtainable from it, on cooling, in rosettes of needles or nodular masses. With reference to *crystallization from alcohol* the following remarks were recorded:

"Während es aus absolutem Alkohol krystallisiert, gewährt es einen interessanten Anblick. Auf der Oberfläche der Lösung bilden sich zahllose Punkte, welche sich zu Körnern, dann zu Rosetten vergrössern und nach mehr oder weniger langem Umherschwimmen untersinken."[2] (Thudichum.)

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[1] Cramer, *Journ. of Physiol.*, xxxi, p. 31, 1904.

[2] These and the succeeding comparative quotations are from the latest descriptions by each author.

"Die Abscheidungen erfolgen in der Regel in zusammenhängenden Massen, die nicht an der Wandung des Glases haften und sich, ohne auseinander zu reissen, in der Flüssigkeit hin- und herbewegen lassen." (Thierfelder.)

With regard to *behavior in water*, agreement was shown as follows:

"Bringt man Phrenosin in Wasser und erwärmt es damit zum Kochen, so wird es weder teigig noch schleimig, sondern schwimmt in losen Flocken in der Flüssigkeit. Da nun das stärkeartige Aufschwellen in heissem Wasser eine Eigenschaft fast aller Phosphatide und Lipotide ist, so kann man aus dem grösseren oder geringeren Erscheinen dieser Eigenschaft an einem gegebenen Präparat die Gegenwart von mehr oder minder Phosphatid oder Lipotid auf leichte Weise ermitteln." (Thudichum.)

"Es ist . . . in Wasser unlöslich, auch nicht darin quellend." (Thierfelder.)

A *color reaction* was referred to as follows:

"Phrenosin giebt eine positive, charakteristische Reaktion mit Vitriolöl. Wenn man es mit dieser Säure verreibt, so wird es zunächst *gelb* und löst sich scheinbar ganz auf. Dann wird es *allmählich purpurrot*, und bei genauem Zusehen findet man, dass die Farbe an ungelöste *Flocken* gebunden ist. Dieselben lassen sich aber durch mehrere Reagenzien wie Chloroform oder Eisessig in Lösung bringen." (Thudichum.)

"Beim Zerreiben von Cerebron mit concentrirter Schwefelsäure tritt *Gelbfärbung* ein, die ungelösten *Flocken* farben sich *allmählich purpurroth.*" (Thierfelder.)

Other duplications of description might be cited.

As to the main reason given by Thierfelder for regarding Thudichum's results with distrust, *i. e.*, "the characteristic lack of exactness" in Thudichum's researches, it is sufficient to say, I think, that the accuracy of Thudichum's work on phrenosin, as shown here, merits respect and consideration. His observations in many other connections have also been repeatedly confirmed by numerous observers, although this could hardly be inferred from Thierfelder's remarks that are quoted on page 175. Errors are inevitable in the work of every investigator, but the mistake that should be the easiest for each of us to refrain from committing is the one of disparaging and discrediting the endeavors of another by such sweeping and undiscriminating condemnation as that to which Thierfelder has subjected Thudichum's

efforts and papers. This must be especially true, it seems to me, for one who finds it necessary (as in the case of Thierfelder's rediscovery of sphingosin, for example, and his complete confirmation of Thudichum's data regarding it) to point directly to results obtained by that particular person twenty-five years before, that were characterized by accurate statement of details and as well as correct conveyance of generalities.

Thierfelder concluded his paper with the following remark:

> "Das Cerebron aber zeigt eine andere Zusammensetzung und einen anderen chemischen Aufbau, als Thudichum für das Phrenosin angegeben hat, und wir können es daher nicht als gerechtfertigt anerkennen, die Entdeckung des Cerebrons Thudichum zuzuschreiben und den von uns zuerst rein (!) dargestellten und genau untersuchten Körper Phrenosin zu benennen." (Page 522.)

I have made it clear, I believe, that the chemical composition of phrenosin, as definitely and deliberately distinguished by Thudichum from various data, in addition to the results of elementary analysis, and recognized apart from a small amount of impurity, was essentially the same as the composition of cerebron. I believe it must be evident that Thudichum's deductions on the composition of phrension from the data furnished by its cleavage products was correct in theory, in spite of the somewhat defective methods employed by him for their quantitative estimation. That he selected a slightly different formula from that preferred by Thierfelder can be a matter of no more consequence, in Thierfelder's estimation than it is in mine, for we both see clearly that pseudocerebrin, for example, to which Gamgee assigned the formula $C_{44}H_{92}NO_8$ is *identical* with cerebron to which Thierfelder assigned the formula $C_{48}H_{93}NO_9$. It is safe to assert that both Thudichum and Gamgee selected incorrect formulas for the same substance. It is not yet certain that Thierfelder, with due recognition of his admirable work upon it, has determined the formula by which the substance can always be correctly indicated.

NOMENCLATURE AND CONCLUDING REMARKS.

After Thierfelder's attention had been called by Gamgee to the obvious fact that pseudocerebrin and cerebron were identical, Thierfelder proposed the retention of the name cerebron in preference to pseudocerebrin, for the following reasons:

"Mit Rücksicht darauf, dass die in Rede stehende Substanz als im Gehirn vorgebildet anzusehen ist, während unter Cerebrinen im allgemeinen Spaltungsprodukte höher zusammengesetzter Gehirnstoffe verstanden werden, halten wir den Namen Cerebron für zweckmässiger und schlagen vor, ihn beizubehalten." (1904, p. 22.)

Unfortunately the term cerebron is easily confused with cerebrin. It seems to me, that, on the ground of prior assignment and because the name phrenosin is more distinctive than cerebron, the latter term should be abandoned and phrenosin retained for the substance. Posner and I suggested, also, for the same reason, that phrenosinic acid would be an improvement on neurostearic acid and cerebronic acid. The question of nomenclature is of no importance, however, compared with prompt recognition of the facts already recited and the consequent permanent placement of phrenosin in its proper chemical as well as historical setting.

I should like, in conclusion, to suggest my belief that no one would be so completely able to settle definitely the facts in this connection, by a comparative study of phrenosin prepared by Thudichum's method and Thierfelder's method, as Prof. Thierfelder himself. I have refrained from undertaking such a study myself because of a desire to leave the way entirely open for a final solution of the question in Prof. Thierfelder's laboratory. It would be personally gratifying to me to find that I have been entirely mistaken in these observations.

I have studied with interest and great profit Prof. Thierfelder's researches, especially those pertaining to protagon and phrenosin. That he has greatly advanced our knowledge of neurochemistry is so well known that it would be presumptuous for me to dwell upon the fact. It was a pleasure on a previous occasion[1] to find that our observations on protagon were in entire harmony with Prof. Thierfelder's.[2] I regret exceedingly that on the matter discussed in this paper, I am unable to agree with Prof. Thierfelder's conclusions.

[1] Lesem and Gies, *Amer. Journ. of Physiol.*, viii, p. 183, 1902; also, Gies and Collaborators, *Biochemical Researches*, i, Reprint No. 11, 1903.

[2] The essentials of those observations on protagon had previously been noted by Thudichum, however, a fact of which I was, at that time, ignorant and to which Thierfelder made no allusion.

GENERAL OBSERVATIONS.

Gamgee and Thierfelder have agreed that pseudocerebrin and cerebron were identical. Kossel and Freytag have shown that their cerebrin, Parcus's cerebrin, and Thudichum's phrenosin were practically the same. Koch has stated his belief that Thudichum's phrenosin, Thierfelder's cerebron, and his own cerebrin were identical. Posner and Gies called attention to the probability that all of these observers were dealing with the same substance.

I have attempted carefully to consider all of Thierfelder's objections to the view, first suggested I believe by Koch,[1] and considered in detail and emphasized by Posner and Gies, that phrenosin, cerebron, and the various cerebrins already named were samples of the same substance. I have been obliged, however, to reiterate the conviction that Thudichum discovered and quite correctly described the essential substance that was associated with slight proportions of impurities in each of these several products. Therefore, I have again expressed the opinion that phrenosin is the most acceptable name for the substance under consideration.

These conclusions in no degree disregard the fact that our knowledge of phrenosin has been very greatly increased by Prof. Thierfelder's admirable studies of it (cerebron.)

ADDENDA.

Shortly after the foregoing paper had been accepted for publication, the first number of volume xlviii of the *Zeitschrift für physiologische Chemie* appeared,[2] with a very brief though significant note at the bottom of page 80, entitled, "Notiz betreff-

[1] Koch's suggestion may be found on page 310 of his paper in volume xi of the *American Journal of Physiology* (1904) and is conveyed in the following sentence: "Phrenosin, $C_{41}H_{79}NO_8$, Thudichum, may be said to be identical or isomeric with the substance isolated by Thierfelder and by Koch, as will be seen from a comparison of the analyses." Following this remark is a table that presents the figures for elementary composition of phrenosin, cerebron, and Koch's own cerebrin.

[2] "Ausgegeben am 19 Mai, 1906."

end das Sphingosin, von F. Kitagawa und H. Thierfelder," as follows:

" Eingehende Untersuchungen über das Sphingosin haben zu dem Resultat geführt, dass diese Substanz *nicht einheitlich ist.*[1] Da die völlige Klarlegung der Verhältnisse bei der Schwierigkeit der Materialbeschaffung und der Trennung noch einige Zeit erfordern wird, so teilen wir dieses Ergebnis schon jetzt mit, um die Angaben des einen von uns zu berichtigen. (*Diese Zeitschrift*, Bd. xliii, S. 21, 1904, und Bd. xliv, S. 366, 1905.")

In the first of the two papers just mentioned sphingosin, precipitated as sulfate from hot alcoholic solution on cooling, was referred to (p. 28) as a substance "welche nach nochmaligem Umkristallisieren rein weiss erhalten wurde, *sich als einheitlich erwies*," etc. The analytic data given on page 29 of the same paper[2] seem to prove it conclusively. No more positive or reliable statement than that just quoted seemed to have been made by Thierfelder in any of his papers regarding either cerebron or cerebronic acid.

In justice to Thudichum, I desire to call attention again to the remarks by Thierfelder that are quoted at the top of page 176 of this paper.

[1] Italics are mine.
[2] By Thierfelder himself.

CHEMICAL STIMULATION OF THE MOTOR AREAS OF THE CEREBRAL HEMISPHERES

By S. S. MAXWELL.

(*From the Rudolph Spreckels Physiological Laboratory of the University of California.*)

(Received for publication, May 9, 1906.)

1.—Introductory Remarks.

The statement that the cerebral cortex can be stimulated chemically depends chiefly upon the experiments of Landois[1] in which certain substances,—creatin, creatinin, disodium phosphate, urinary sediment, etc.—in powdered form or in solutions of various concentrations, were placed directly upon the brain surface. He saw after an interval of five minutes or longer the beginning of certain effects—bending of the body axis to the opposite side, clonic contractions of the chewing muscles, followed by rapidly repeated single contractions of the nose, ear, and eye muscles on the opposite side. Then the whole fore part of the body, including the fore limbs, fell into quivering. Strong cramps also involved the muscles on the operated side, but less strongly than on the crossed side.

Bickel[2] repeated and confirmed the observations of Landois. He admits indeed that the conditions of his experiments make it impossible to decide whether only the gray matter or the white matter also had been stimulated, and that all one can affirm with certainty is that on account of the shortness of the latent period—ten to fifteen minutes—the stimulation could have extended only to the "peripheral layer" of the hemisphere, and not to those more deeply lying subcortical centers whose excitation would have evoked bilateral instead of unilateral contractions.

Regoli[3] applied solutions of barium chloride to the motor area

[1] Landois, *Deutsch. med. Woch.*, xiii, p. 685, 1887; and *Wien. med. Presse*, xxviii, p. 223, 1887.

[2] Bickel, *Arch. f. d. ges. Physiol.*, lxxii, p. 214, 1898.

[3] Regoli, *Boll. della Soc. tra i cultori di Sc. Med. Nat. in Cagliari*, 1899–1900, p. 151.

of the dog's brain and saw in a short time (ten minutes in one experiment, and in another the exact time is not stated) the occurrence of spontaneously appearing epileptic seizures on the opposite side of the body. This author as well as Sabbatani[1] and Roncoroni applied certain solutions, especially of the calcium precipitants, to the brain surface, and by testing with electrical stimulation after ten-minute intervals, were able in most instances to demonstrate an increased excitability.

Gallerani and Lusanna[2] applied Liebig's meat extract to the brain surface and after an interval of $1\frac{3}{4}$ hours in the dog and $\frac{3}{4}$ hours in the rabbit were able to produce a sort of reflex epilepsy by the stimulation of peripheral nerves. Experiments with meat extracts were also reported by Koranyi and Tausczk.[3]

From these and similar experiments the conclusion has been drawn that the cortex had been stimulated chemically, and this conclusion has been made use of in the explanation of eclampsia, uræmic convulsions, the nervousness of pregnancy,[4] etc.

As I have stated elsewhere[5] these experiments in my opinion are open to objection as evidence of chemical stimulation of the cortex because, in the relatively long period between the application of the substances and the occurrence of the contractions, powdered substances or strong solutions would have time to act osmotically by extracting water from the tissues, or else through diffusion to act upon the underlying white matter of the corona radiata. Moreover, the substances used by Landois, with the exception of the phosphate, have not been shown to act specifically as nerve stimulants.

We know through the experiments of Loeb[6] that chemical stimulation can be best brought about through the action of those substances whose anions precipitate calcium, and his experiments as well as those of many other observers have proved that the relation is general. The knowledge of this fact gives us a definite method for the application of chemical stimula-

[1] Sabbatani, *Arch. ital. de biol.*, xliv, p. 398.

[2] Gallerani and Lusanna, *ibid.*, 1891.

[3] *Internat. klin. Rundsch.*, 1890.

[4] Blumreich and Zuntz, *Arch. f. Physiol.*, 1901, Suppl., p. 266.

[5] *Univ. of California Publications, Physiology*, iii, p. 17, 1906.

[6] Loeb, *Amer. Jour. of Physiol.*, v, p. 362, 1901; also in *Studies in General Physiology*, Chicago, ii, p. 692, 1905.

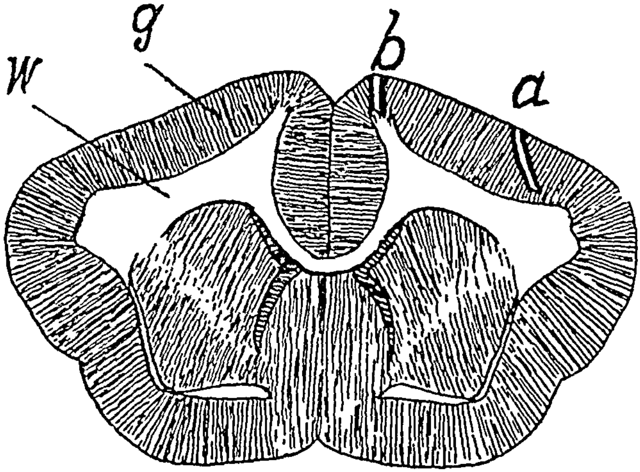
tion; namely, in the use of such substances as the soluble citrates, oxalates, fluorides, oleates, etc. In attempting to make use of this method in the investigation of certain areas of the cortex I found that I could not get any evidence whatever of chemical stimulation of the gray matter but that stimulation occurs very promptly when solutions of the calcium precipitants are injected so deeply as to act directly upon the white matter.

Most of my experiments were performed upon the motor areas of the cerebral cortex of the rabbit, but the cat was used in a sufficient number of cases to prove that the results are precisely the same. At the beginning of each experiment the localization of the various motor areas and the degree of excitability were determined by electrical stimulation with a weak interrupted current and similar tests of the excitability were made at intervals during the progress of the experiment. Care was taken to avoid excessive cooling or drying or any other factor known to be liable to cause a lowering of sensibility. It is well known that deep narcosis through ether greatly lowers or totally abolishes the response to electrical stimulation. Morphine except in very heavy doses interferes less than ether with the excitability of the brain tissue. Some of my experiments were made under light etherization. In others morphine alone was used. In order, however, to set aside completely the question of narcotic effects, in a large number of the experiments local anesthesia alone was employed. This was secured by the injection of a very small quantity of a one-half per cent. solution of cocaine into the skin of the animal along the line of incision. The controls by electrical stimulation showed perfectly the absence of narcotic interference.

2.—*Application of Substances to the Brain Surface.*

When the powdered substances were placed directly upon the brain surface I in no case observed results which could be fairly interpreted as stimulation of the cerebral cortex. I could indeed confirm the observations of Landois upon the effect of creatin and creatinin so applied; but the latent period was usually considerable—fifteen minutes to over half an hour. Moreover I was also able by the use of crystals of cane sugar upon the surface of the motor region of one side of the brain, to produce after

sodium citrate and $\frac{M}{3}$ and $\frac{M}{6}$ sodium oxalate. Whether stimulation did or did not occur seemed to depend upon two factors; namely, the depth of the injection and the thickness of the cortex at that particular point. When stimulation did occur the response came very promptly, within a few seconds at most, and the localization was as precise as in the case of electrical stimulation. In all my experiments not a single case occurred of this prompt and definitely localized response where the puncture did not reach the innermost layer of the gray matter. This difference is well shown in the accompanying figure of a section through two punctures. That at *a* gave no indication of stimulation; that at *b*, although not so deep, reached the white matter and caused extension of the foreleg and spreading of the toes.



DESCRIPTION OF DIAGRAM.—Frontal section of brain of rabbit at level of two injections of $\frac{M}{8}$ sodium oxalate. That at *a* gave no response, that at *b* extension of forelegs and spreading of toes. The shaded areas (*g*) represent gray matter; the unshaded (*w*), white matter.

Before going into more detailed description of the experiments it is necessary to mention certain possible sources of error:

(*a*) Mechanical Stimulation.—The insertion of the needle might cause mechanical stimulation. In order to exclude this effect the needle was first inserted to the desired depth and the liquid was not passed in until a moment later. In this way it could be determined that the stimulation was due to the

solution and not to the pressure of the needle. Stimulation caused by the needle alone seldom occurred and then only when the puncture was unnecessarily deep.

It was also possible that the effect was due to the pressure of the injected liquid. The smallness of the amount, two or three drops at most, would make this improbable. The more decisive evidence lies in the fact that injection of $\frac{M}{8}$ sodium chloride caused no response while $\frac{M}{8}$ sodium citrate brought about contractions.

The introduction of the needle ruptures blood-vessels and the wound becomes filled with blood. The pressure of liquid in the wound, however, could not be greater than the pressure to which the brain matter is subjected by the blood in the vessels, so that under the conditions of the experiment the hemorrhage could not stimulate through mechanical pressure.

(*b*) Osmotic Stimulation.—Where solutions of a concentration sufficient to give a higher osmotic pressure than that of the blood serum were employed, as was done in a number of the experiments, the stimulation might have been due to the withdrawal of water. That osmotic stimulation can be readily brought about is shown by the fact that injection of $\frac{M}{3}$ sodium chloride causes contractions while an $\frac{M}{8}$ solution of the same salt does not. But the fact that sodium citrate and sodium oxalate stimulate in solutions practically isotonic with the blood serum must depend upon chemical and not upon osmotic action.

In the experiments here described about forty animals were used and a number of trials were made on each animal. Extracts from the notes on a few of these may serve as examples:

March 13, 1906. Rabbit; local anesthesia only; right motor area exposed; areas for left foreleg, mouth, and neck determined by electrical stimulation; injections of $\frac{M}{3}$ sodium oxalate.

Puncture 1. Needle inserted, no response; injection, no response.
Puncture 2. Needle inserted, no response; injection, no response.
Puncture 3. Needle inserted, no response; injection, strong movement of left foreleg.
Puncture 4. Needle inserted, no response; injection, no response.
Dissection of brain, March 15, showed:
Puncture 1. Not nearly to bottom of gray matter.
Puncture 2. Not nearly to bottom of gray matter.
Puncture 3. Reached to surface of white matter.
Puncture 4. Not quite to bottom of gray matter.

The above experiment is in accord with all the others in that stimulation did not occur unless the solution reached at least to the inner surface of the gray matter. It does not however distinguish between osmotic and chemical stimulation. In the following experiment the effect must have been chemical and not osmotic:

March 17, 1906. Rabbit; local anesthesia only. Motor areas of right hemisphere determined as usual. Injection of $\frac{M}{8}$ sodium oxalate mixed with India ink.

Puncture 1. No response.
Puncture 2. No response.
Puncture 3. Contraction of muscles on left side of snout.
Puncture 4. Slight movement of foreleg.
Puncture 5. No response.
Puncture 6. No response.
Responses to electrical stimulation good at close of experiment.

Sections of brain, March 19. showed that punctures 3 and 4 reached to the white matter; all the others terminated in the gray matter.

I have stated that in no instance did stimulation result from injection into the gray matter only, but that stimulation occurs very promptly when the solution reaches the white matter. I must mention that in a few exceptional cases no response was obtained to the deeper injection. The following was one of the most marked instances of this sort:

March 19, 1906. Rabbit; local anesthesia; right motor area determined electrically; injection of $\frac{M}{7}$ sodium citrate mixed with India ink.

Puncture 1. No response.
Puncture 2. No response.
Puncture 3. Movement of left side of snout.
Puncture 4. No response.
Puncture 5. No response.
Section, March 29.
Puncture 1. Terminates in gray matter.
Puncture 2. Almost to white matter.
Punctures 3, 4, and 5. All reached to white matter.

It is well known that barium salts act powerfully as nerve stimulants. Experiments with barium chloride gave results exactly parallel to those obtained with the calcium precipitants. Stimulation did not occur when strong solutions were placed upon the gray matter; contractions resulted only after the lapse

of an interval apparently long enough to allow diffusion to deeper levels. Injection of a solution isosmotic with the blood serum stimulated promptly only when the white matter was reached.

5.—*Discussion of Results.*

The results of these experiments justify the conclusion that the gray matter is not irritable to chemical stimulation. The same conclusion holds for osmotic stimulation. I have not observed a single fact which could be interpreted as an indication of stimulation of the gray matter by either of these methods. As I have already pointed out, the observations which other authors have described as evidence of chemical stimulation of the cortex are based upon effects occurring after an interval of many minutes, while my observations show that chemical and osmotic stimulation of the white matter can occur within a very few seconds. If this does not prove, as I believe it does, that the stimulation takes place in the white and not in the gray matter, it must at least prove that the gray matter is far less irritable than the white matter.

It was proved through the experiments of Goltz[1] that the cortex is devoid of irritability to mechanical injury. While removing the gray matter of the cortex of the motor area he often saw movements of the limbs of the opposite side. He experimented to discover whether these movements were produced by more superficial or by deeper injuries and established the fact that contractions occurred only when the instruments reached a depth of at least four millimeters (in the dog's brain); that is to say, a depth sufficient to reach the white matter. Professor Loeb[2] in his experiments on the brain confirmed these observations and found like Goltz that when the instruments touched the white matter stimulation promptly occurred. The absence of sensibility in the gray matter of the cortex is also thoroughly well known through clinical and surgical experience.

Since the gray matter of the cortex is inexcitable to mechanical, chemical, and osmotic stimulation, the only evidence for its irritability rests upon the effects of electrical stimulation. To

[1] Goltz, *Arch. f. d. ges. Physiol.*, xxvi, p. 37. 1881.
[2] Personally communicated.

this evidence it has long been objected that on account of the spreading of the lines of force it is not possible to say at what level the excitation really occurs. Many attempts, like that of Bubnoff and Heidenhain,[1] have been made to set aside this objection but none of them have directly or unequivocally proved the electrical irritability of the cortex. Moreover it is well known that when electrodes are placed upon the surface of the motor area the strength of the stimulus necessary to produce a muscular response bears a pretty direct relation to the thickness of the cortex. The cat requires a stronger current than the rabbit, the monkey a stronger current that the cat or dog. This matter is well summarized in the following statement by Schäfer[2]:

> " Both in the orang and in man a much greater strength of current than that employed for lower animals is needed to provoke movements, a result which appears to indicate that they are caused by excitation not of the superficial but of the more deeply lying parts of the cortex which is much thicker than that of the monkey. The convolutions are also more excitable near their convexity than near their bounding fissures."

If by the expression "the more deeply lying parts of the cortex" one is to understand the white matter, then the results of chemical, osmotic, mechanical, and electrical stimulation are in complete accord.

Furthermore it seems probable from the work of Loeb that electrical stimulation is only a form of chemical stimulation.[3] For he has shown that a nerve entirely insulated and placed parallel to the spark discharge of a Toepler-Holtz machine will cause contractions whenever a spark is produced, while no or only a minimal effect is produced if the nerve is placed at right angles to the spark discharge. Since in this case no electrical charges are withdrawn from the ions, the stimulation can only be due to localized changes in the concentration of the ions. But the results are the same as if the electrodes had been placed upon the nerve, hence in this case also the contractions are

[1] Bubnoff and Heidenbain, *Arch. f. d. ges. Physiol.*, xxvi, p. 167, 1881.

[2] Schäfer, *Text Book of Physiology*, Edinburgh and London, ii, p. 748, 1898.

[3] Loeb, *Arch. f. d. ges. Physiol.*, lxvii, p. 483, and lxix, p. 99, 1897; reprinted in *Studies in General Physiology*, ii, p. 482, 1905; *Univ. of California Publications, Physiology*, iii, p. 9, 1906.

caused by changes in the concentration of the ions. He has also shown that the increase of irritability at the cathode can be imitated by immersing a nerve or muscle in a solution of a sodium salt whose anions tend to precipitate calcium, *e.g.* the oxalate, citrate, fluoride, phosphate, etc., and that when this condition is produced the tissue may be restored to normal or lowered irritability by adding a sufficient quantity of calcium (or magnesium) chloride.[1] It follows that an increase of free calcium (or magnesium) ions causes a condition of diminished irritability, corresponding to the anelectrotonus caused by the galvanic current, while a decrease in the concentration of these ions brings about a condition similar to the catelectrotonus caused by the galvanic current. He then raised the question whether the effect of the current could be to increase the concentration of the free calcium (and magnesium) ions at the anode and decrease it at the cathode; and showed that this is probable from the following considerations[2]: Changes in concentration of the various ions in the neighborhood of the electrodes depend primarily on their migration velocities. For the anions occurring in muscle and nerve this velocity varies very considerably; the chlorine ion has a relatively high velocity (65.4), while the anions of the organic acids which must be present, such as oleate, stearate, palmitate, and lactate, have a very small velocity, probably below 30. On account of these differences in the velocity of the ions, more chlorine ions than oleate, palmitate, etc., must leave the neighborhood of the cathode in a unit of time, and hence in the region of the cathode the effect of the current must be to bring about a relative increase in the concentration of the calcium precipitants, that is, an effect of the same nature as that which occurs when the nerve is placed in a solution of sodium oxalate, citrate, fluoride, etc. On the other hand a relatively larger proportion of the chlorine ions must migrate to and collect at the anode in a unit of time, thus increasing the concentration of the very soluble and highly dissociated calcium chloride.[3]

[1] Loeb, *Amer. Journ. of Physiol.*, v, p. 362, 1901; reprinted in *Studies in General Physiology*, ii, p. 692, 1905.

[2] Loeb, *Univ. of California Publications, Physiology*, iii, p. 13, 1905.

[3] Nernst has also by another method arrived at the conclusion that an electric current stimulates only by causing changes in the concentration

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[2] Loeb, *Univ. of California Publications, Physiology*, iii, p. 13, 1905.

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If then it be true, as I believe the above considerations clearly demonstrate, that electrical stimulation is of the same nature as chemical stimulation, it is highly improbable that the gray matter is excitable to the one and inexcitable to the other.

Bethe's[1] experiments on the nervous system of the crab seem to show that reflexes can occur without the intervention of the cell bodies of the ganglion cells. In my experiments on the cortex it has, of course, been impossible to discriminate between cell bodies and fibers. But this much is clear, that the cortex, composed in large proportion of cell bodies, is either far less irritable than the white matter, composed mainly of fibers, or is wholly unirritable.

SUMMARY.

Briefly summarized the results of my experiments are as follows:

1. Substances applied to the surface of the cortex either give no indication of stimulation, or do so after so long an interval that they would have time to act osmotically or by diffusion upon the underlying white matter.

2. The white matter of the motor areas can be stimulated chemically by the calcium precipitants and by barium chloride in solutions isosmotic with the blood serum. The response to such stimulation is very prompt, occurring within a few seconds at most, after application of the solution. The same substances when applied in the same concentration to the cortex give no result at all or only after an interval of some minutes.

3. Solutions of high concentration can stimulate the white matter by osmotic action very promptly and effectively.

4. When solutions are injected into the gray matter but not so deeply as to reach the white matter no evidence of stimulation is seen. The gray matter is apparently devoid of irritability to chemical and osmotic stimulation as well as to mechanical and electrical stimulation.

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of the ions. Nernst, *Nachricht. d. Gesellsch. d. Wissensch. z. Göttingen*, p. 104, 1899, and Nernst and Barratt, *Zeitschr. f. Electrochem.*, x, p. 664, 1904.

[1] Bethe, *Biolog. Centralbl.*, xviii, p. 843, 1898.

THE INFLUENCE OF TEMPERATURE UPON THE CONTRACTION OF STRIPED MUSCLE AND ITS RELATION TO CHEMICAL REACTION VELOCITY.

By THEO. C. BURNETT.

(*From the Rudolph Spreckels Physiological Laboratory, University of California.*)

(Received for publication, May 12, 1906.)

That temperature influences muscular contraction is a fact that has long since been accepted by physiologists. That the process or processes underlying muscular contraction are chemical in their nature is pretty generally accepted, although to my knowledge nothing has been done to bring these processes into accord with the physical chemical fact that for every ten degrees' rise of temperature the velocity of a reaction is doubled or trebled.

In his lectures on physical chemistry, Cohen [1] quotes the results of experiments made by Clausen [2], on the production of carbon dioxide by germinating seeds and points out that between 0° and 25°, the amount of carbon dioxide expired "increases in all cases about 2.5-fold." He also takes the results of Hertwig's [3] experiments on the influence of temperature upon the rate of development of the eggs of *Rana* and gets similar results. Abegg [4] gives the results of experiments made at his suggestion by Dr. Peter, on the eggs of *Echinus microtuberculatus*, and of *Sphærechinus granularis*. Later [5] he quotes a table of carbon dioxide production in frogs. His quotients are about 2. R. O. Herzog [6] has obtained similar results for yeast.

[1] Cohen, E., *Physical Chemistry for Physicians and Biologists.* Translated by Fischer, 1903.

[2] Von Clausen, *Landwirtsch. Jahrb.*, xix, p. 893.

[3] Hertwig, O., *Arch. f. mikroskop. Anat. u. Entwicklungsgesch.*, li, p. 319.

[4] R. Abegg, *Zeitschr. f. Elektrochem.*, xxxiii, p. 528, 1905.

[5] *Ibid.*, xlvi, p. 823, 1905.

[6] R. O. Herzog, *ibid.*, xlvi, p. 820, 1905.

It has been an idea of Prof. Loeb's for some time that this same method might be applied to other physiological phenomena. In the case of the heart-beat, for instance, he reasoned that if the process underlying it was chemical, the number of beats in a given time ought to be doubled, or nearly so, for each ten degrees' rise of temperature. In order to determine this, Snyder[1] undertook, at Dr. Loeb's suggestion, some experiments on the heart of the Pacific Terrapin. He found that when such a heart was subjected to various temperatures, its beats were accelerated in an orderly manner, his figures showing that for every ten degrees rise of temperature the heart-beat was about doubled, corresponding to the formula of van't Hoff[2] and Arrhenius,[3] $\frac{\text{Velocity at } Tn + 10}{\text{Velocity at } Tn} = 2$ or 3, where T = temperature.

It at once became evident that in all probability striped muscle would behave in a similar manner; and in order to test it, I undertook some experiments on frog's muscle with the following results:

The gastrocnemius muscle of the frog was mainly used, although others (sartorius, triceps femoris) have been used with similar results. The muscle was prepared in the usual manner (without the sciatic in the case of the gastrocnemius) and attached to a recording lever, weighted with 10 grams (5 grams for sartorius). Platinum wire electrodes were attached to the extremities of the muscle, which was stimulated directly by a break induction shock of medium intensity, a "knock-over" key being attached to the upright of the kymograph. A pin projecting from the lower margin of the drum served to knock over the key, which was in the primary circuit. A Du Bois-Reymond key was in the secondary circuit. The kymograph used was of the Cambridge pattern. The muscle was immersed in a solution of sodium choloride made up with tap water, of $\frac{N}{8}$ concentration, and this solution was kept at

[1] Snyder, Chas. D., *Univ. of California Publications, Physiology*, ii, p. 125.

[2] Van't Hoff, J. H., *Vorlesungen über theoretische u. physikalische Chemie*, 1898, i, p. 224.

[3] Arrhenius, S., *Zeitschr f. physik. Chem.*, iv, p. 226, 1899.

various temperatures ten degrees apart within the limits of safety. According to the size of the muscle, it was allowed to remain from three to five minutes in the solution at a given temperature before stimulating. The time tracing was made with a tuning fork vibrating one hundred to the second; drum rapid. One Edison–LaLande cell furnished the current.

The length of the latent period was made the basis of calculation, the shorter the latent period the more rapid being the reaction. Obviously, as we are here dealing with a time interval, the formula $\frac{\text{Velocity at } Tn + 10}{\text{Velocity at } Tn} = 2 \text{ or } 3$ must be reversed in order to obtain quotients that will be comparable with the observations of physical chemists, and the formula for our purpose becomes $\frac{\text{Latent period at } Tn}{\text{Latent period at } Tn + 10} = 2 \text{ or } 3.$

The table which follows gives the results of fifteen experiments and is considered all that is necessary, as a description of the technique is really a description of the experiments, and muscle curves are too familiar to physiologists for me to dwell upon them here. Under the head of remarks it will be noticed that muscles were sometimes used two days in succession, and regardless of their condition. This was done purposely in order to ascertain what difference, if any, the age of the muscle would give rise to. As will be seen, about the only difference was in a lengthening of the latent period; the quotients do not differ markedly. Experiments 8 and 9 were made in a muscle warmer instead of the tap-water saline solution.

No of Experiment	Temperature	Latent Period in Seconds	Ratio	Remarks
1	5°C	0225		Gastrocnemius of frog.
"	15°"	0161	1 4	
"	25°"	0073	2 2	
2A	5°C	038		Same muscle as Ex. 1 having been in tap-water saline 24 hrs.
"	15°"	012	3 17	
"	25°"	.0086	1 4	
2B	10°C	023		Same muscle as Ex. 1 having been in tap-water saline 24 hrs.
"	20°"	01	2 3	
"	30°"	0083	1 2	

No. of Experiment.	Temperature.	Latent Period in Seconds.	Ratio.	Remarks
2C	3°C	.034		The gastrocnemius was from a frog that had been pithed the day before.
"	13°"	.0205	1.66	
"	23°"	.0077	2.66	
"	33°"	.006	1.28	
3A	3°C	.0165		
"	13°"	.0094	1.76	
"	23°"	.0062	1.52	Fresh gastrocnemius.
"	33°"	.0037	1.68	
3B	10°C	.0087		
"	20°"	.0062	1.41	Fresh gastrocnemius.
"	30°"	.0037	1.68	
4	10°C	.0177		Same muscle as in 3B after 24 hrs. in tap-water saline.
"	20°"	.0068	2.6	
"	30°"	.0055	1.24	
5A	3°C	.0231		
"	13°"	.0138	1.67	
"	23°"	.0062	2.23	Fresh gastrocnemius.
"	33°"	.005	1.24	
"	10°"	.0137		
"	20°"	.0075	1.83	
"	30°"	.0058	1.29	
5B	5°C	.0238		
"	15°"	.01	2.38	
"	25°"	.0053	1.89	Fresh gastrocnemius.
"	35°"	.0062	0.85	
6	3°C	.0687		
"	13°"	.016	4.29	Same muscle as in 5A and B after 24 hours in tap-water saline.
"	23°"	.0065	2.46	
"	33°"	.005	1.3	
"	10°"	.0165		
"	20°"	.0083	1.99	
"	30°"	.005	1.66	
7	2°C	.0825		
"	12°"	.036	2.29	
"	22°"	.0237	1.52	Fresh gastrocnemius.
"	32°"	.01	2 37	
8	3°C	.036		
"	13°"	.01	3.6	This gastrocnemius was in poor condition having a large spot of ecchymosis.
"	23°"	.0057	1.75	
"	33°"	.0057	1.0	
9	5°C	.0537		
"	15°"	.0184	2.9	
"	25°"	.0092	2.0	Triceps femoris.
"	35°"	.006	1.55	

No. of Experiment.	Temperature	Latent Period in Seconds.	Ratio.	Remarks.
10A	5°C	.0472	2.36	Sartorius.
"	15°"	.02	1.67	
"	25°"	.012	1.0	
"	35°"	.012		
10B	2°C	.56	2.4	Sartorius.
"	12°"	.0255	1.65	
"	22°"	.0155	1.13	
"	32°"	.0137		
11	5°C	.045	4.09	Triceps femoris muscle. This muscle was unavoidably exposed about ½ hr. to temp. of 5°; the latent period as is seen was very much prolonged
"	15°"	.0111	2.22	
"	25°"	.005	0.57	
"	35°"	.0087		
12	2°C	.37	2.2	Gastrocnemius.
"	12°"	.0168	1.53	
"	22°"	.011	1.26	
"	32°"	.0087		
13	2°C	.066	2 2	Same as above.
"	12°"	03	1.76	
"	22°"	.017	1.42	
"	32°"	.012		
14	3°C	.0987	4 39	Triceps femoris.
"	13°"	.0225	1.87	
"	23°"	.012	1.71	
"	33°"	.007		
15	3°C	.0622	1.48	Gastrocnemius.
"	13°"	.042	2 89	
"	23°"	.0145	1.38	
"	33°"	.0105		
15A	2°C	.0342	1.28	Gastrocnemius.
"	12°"	.0268	2.23	
"	22°"	.012	1.48	
"	32°"	.0081		
15B	5°C	.0394	1.86	Same as above.
"	15°"	.0212	2.12	
"	25°"	.01	1.42	
"	35°"	.007		
15C	10°C	.0292	1.81	Same as above.
"	20°"	.0161	1.46	
"	30°"	.011		

In order to gain a more comprehensive view of these re-

sults, the quotients are placed in columns in the following table.

TABLE OF QUOTIENTS.

Centigrade.

2–12	3–13	5–15	10–20	12–22	13–23	15–25	20–30	22–32	23–33	25–35
2.29	1.66	1.4	2.3	1.52	2.66	2.2	1.2	2.37	1.28	—
2.19	1.77	3.17	1.4	1.65	1.52	1.4	1.68	1.13	1.68	—
2.2	1.67	2.38	2.6	1.53	2.23	1.9	1.24	1.26	1.24	—
2.2	4.29	2.9	1.83	1.76	2.46	2.	1.29	1.42	1.3	.85
1.23	3.6	2.36	1.99	2.23	1.75	1.67	1.66	1.48	1.	1.53
	4.35	4.05	1.81		1.87	2.22	1.46		1.71	.57
	1.48	1.86			2.89	2.12			1.38	1.43

Making allowance for experimental error due to the crudeness of technique where such delicate reactions are involved, a glance at the above table shows that within certain narrow limits of temperature, from 10° to 25°, the figures approximate those of observers in other fields. In only two instances do the figures go below 1.5 and in most cases they are close to 2 or between 2 and 3. Above 25° the quotients fall off noticeably, reaching a minimum at 35°. Below 10° there is considerable irregularity, the quotients ranging from 1.4 to 4.35. These high figures are due to the enormous lengthening of the latent period at low temperatures, and when one considers that a rise of the ten degrees brings the muscle within what might be termed the optimum limits of temperature, the wonder is that all the quotients are not high between 2° and 12° and 3° and 13°. Then, too, the condition of the muscle may have something to do with it. In Experiment 2 A (3.17) and Experiment 6 (4.29), the muscles were twenty-four hours old. In Experiment 8 (3.6) the muscle had evidently been bruised. In 11 (4.09) and 14 (4.39), the triceps was from a frog that had been pithed several hours before and had been exposed carelessly while the gastrocnemius was being used. These facts are given simply for what they are worth—a suggestion of a possible cause for the high quotients. As these figures do not appear under any other conditions than low temperature and doubtful condition of the muscles, it seems a plausible explanation.

The question naturally suggests itself, "Is the latent period

constant for all muscles at a given temperature, or does it vary with the individual muscle?" At first glance it would seem as if it might be so, but on closer inspection the differences in the latent period not only of different muscles but of the same muscle at different times renders a negative answer necessary, tentatively at least.

In conclusion it would seem quite certain that the fundamental principle underlying the contraction of striped muscle is a chemical one and that its reaction velocity is influenced by temperature much in the same way as are other chemical reactions. One might go further and point out that cold retards this reaction, that within certain limits a rise of temperature approximately doubles its velocity, which again falls with a further increase of temperature. Is not this analogous to a ferment?

I wish to thank Dr. Loeb for his kindly interest and also the various members of the Physiological Department for their helpful suggestions from time to time.

METABOLISM EXPERIMENTS WITH ORGANIC AND INORGANIC PHOSPHORUS.

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(Received for publication, May 12, 1906.)

Since Kossel[1] first intimated that organic phosphorus was of importance as a food for the organism, considerable work has been undertaken by various scientists to determine the rôle which organic and inorganic phosphorus respectively play on the digestibility of the proteids and on the amounts of phosphorus absorbed and retained by the body.

Röhmann[2] and his school (Marcuse,[3] Steinitz,[4] Leipsiger,[5] Zadik,[6] *et al.*) have all proceeded on the hypothesis that the organism is incapable of forming synthetically the phosphorus-containing proteids necessary for the life of the cells out of phosphorus-free proteids and inorganic phosphates. Although these experimenters have produced a mass of evidence tending to corroborate their hypothesis, they have been opposed by Keller,[7] who was able to obtain a plus phosphorus balance on feeding a phosphorus-free food together with inorganic phosphates, and by Gevärts,[8] who stated that only the phosphates of the food can be absorbed and that from these phosphates the cells of the body synthesize lecithin, nuclein, and other phosphorus compounds

The authors being especially interested in the development and production of cereals, plants which contain phosphorus in considerable quantities, it seemed desirable to study the rôle which this phosphorus plays in nutrition.

[1] *Monatsh. f. Geburtshilfe u. Gynaekol.*, i, p. 175.
[2] *Berl. klin. Wochenschr.*, No. 36, p. 789, 1898.
[3] *Arch. f. d. ges. Physiol.*, lxiv, p. 223, 1896.
[4] *Ibid.*, lxxii, p. 75, 1898.
[5] *Ibid.*, lxxviii, p. 402, 1899; *Inaug. Dissert.*, Breslau, 1900.
[6] *Arch. f. d. ges. Physiol.*, lxxvii, p. 10, 1899.
[7] *Arch. f. Kinderheilk.*, 1900.
[8] *Jahresber. f. Thierchem.*, xxxi, p. 732, 1901.

According to the recent work of Hart[1] and Andrews, the phosphorus occurring in cereals is almost entirely in the organic form, *e. g.*, in wheat as a calcium-magnesium-potassium compound of anhydrooxymethylenediphosphoric acid, first isolated by Posternak.[2] When it is considered that approximately one per cent. of wheat consists of phosphoric acid and that this same acid in wheat-bran amounts to over three per cent., and that this large quantity of phosphoric acid united to calcium-magnesium-potassium as an organic compound is virtually treated as a refuse product in so far as human nutrition is concerned, thereby robbing the organism of a valuable food constituent, then it becomes of importance to investigate the rôle played by this phosphorus in metabolism. For that purpose it was decided to conduct feeding experiments, using both rabbits and dogs, as representatives of the herbivorous and carnivorous families respectively, feeding them phosphorus in the form of disodium phosphate and then again in various organic forms such as occur in wheat-bran and in egg-yolk.

Two rabbits were selected, one a black female weighing about 1600 grams, the other a white male whose weight at the beginning of the experiment was 2500 grams.

Each rabbit was confined in a suitable wire cage which allowed the feces and urine to be easily separated. The experiments covered five-day periods with three distinct kinds of food:

I. (a) Normal food: oats, corn, carrots.
   (b) Oats, corn, carrots plus inorganic phosphorus.
   (c) Oats, corn, carrots plus organic phosphorus.

II. Artificial food or phosphorus-poor food (edestin, crackers, carrots, starch, sugar):
   (a) Plus organic phosphorus.
   (b) Plus inorganic phosphorus.

III. (a) Normal bran plus carrots and starch.
   (b) Phosphorus-poor bran[3] plus carrots, starch, and inorganic phosphorus.

[1] *N. Y. Agr. Exp. Sta. Bull.*, 238.

[2] *Rev. gén. de botan.*, xii, p. 5, 1900.

[3] This was ordinary wheat-bran which had been extracted with 0.2 per cent. HCl. The extracted bran thus obtained contained about 0.3 per cent. of P_2O_5.

(c) Phosphorus-poor bran plus carrots, starch, and organic phosphorus.

In all experiments one gram of salt mixture (consisting of 450 grams of sugar, 4 grams of $CaCl_2$, 15 grams of NaCl, 30 grams of KCl, and 1 gram of $MgSO_4$) was given per diem.

After bringing the rabbits to a nitrogenous equilibrium, the experiments were commenced. The feces were collected at frequent intervals during the day, owing to the fact that the rabbits persistently eat them. The urine and food residues were collected daily. All these samples were composited and analyzed: the nitrogen in the foods, feces, and urine according to the Gunning method [1] and the phosphoric acid in the food and feces by Neumann's [2] method; the phosphoric acid in the urine was determined by both the uranium acetate method and the method of fusion with sodium hydroxide, precipitation with ammonium molybdate, and titration of the yellow precipitate.

In this way a check on the nitrogen and on the phosphoric acid in the urine was obtained, and besides this check the double determination of phosphoric acid served as an indication of the presence of any organic phosphorus that might be found therein.

In order to show the the difference between the results obtained by the uranium acetate and the fusion methods of determining phosphorus in urine, the following average figures obtained during eight periods of feeding the rabbits and six periods of feeding the dog, during which phosphorus was added either in the organic or the inorganic form, are given below:

Subject	Average Amounts of P_2O_5 in Urine per Period.			
	Uranium Acetate Method Grams P_2O_5	Fusion Method Grams P_2O_5	Difference	
			Grams P_2O_5	Per cent.
Black rabbit (8 periods)	1 340	1.395	.055	4
White rabbit (8 periods)	1.780	1.833	.053	4
Dog (6 periods)	3.284	3.377	.093	3

These results show that from three to four per cent. of the

[1] All the nitrogen determinations were carried out by Mr. T. C. Trescot.

[2] *Zeitschr. f. physiol. Chem.*, xxxvii, p. 115, 1902.

phosphorus in the urine appears to be in the organic form. When it is considered, however, that the variation of results by the two methods is no greater when organic phosphorus is fed than when inorganic phosphorus is fed and that these results would in any case be taken as good duplicates, we do not feel warranted in accepting the view that normal urine contains organic phosphorus. Bergmann[1] feeding glycerophosphoric acid was unable to find any organic phosphorus in the urine, the organic phosphorus being all broken up and excreted as an inorganic salt.

Likewise a check on the nitrogen content of the urines was obtained by determining the nitrogen in the daily, and composite samples, the results being as follows:

Subject	Average Amounts of Nitrogen in Urine per Period.		
	Amounts from Composite Sample Grams	Amount from Daily Sample Grams	Difference Grams
Black rabbit (7 periods)	4.01	3.99	+.02
White rabbit (7 periods)	5.57	5.58	—.01
Dog (5 periods)	9.54	9.69	—.15

It appears from the above that in the case of the urines from rabbits, it is necessary only to determine the nitrogen in the composite sample. It is otherwise, however, with dog urine. Here there is an appreciable loss of nitrogen on standing, a loss equal to 1.5 per cent. This is due no doubt to the character of the food, giving rise in the urine to bodies more easily decomposable, *e. g.*, urea, ammonia, etc. In no case was any preservative added to the urine.

The amounts of nitrogen and phosphoric acid found in the food residues were subtracted from those of the food given, so that the figures given in the tables represent only the nitrogen and phosphoric acid ingested.

EXPERIMENT I, a, b, c.

In Experiment I, a, the black rabbit received per diem 35

[1] *Arch. f. exper. Path. u. Pharm.*, xlii, p. 77, 1902.

grams of oats, 15 grams of corn, and 35 grams of carrots; the white one getting 50 grams of oats, 20 grams of corn, and 50 grams of carrots. Each was given one gram of salt mixture daily.

In Experiment I, b and c, the same basal food was given as in I, a. In I, b, however, an added amount of inorganic phosphoric acid[1] in the form of a mixture of sodium dihydrogen phosphate and disodium hydrogen phosphate was given equal to 1.157 grams, and in I, c, 1.258 grams of phosphoric acid were given to each rabbit in the form of the calcium-magnesium-potassium salt of anhydrooxymethylenediphosphoric acid obtained from wheat-bran. The results obtained are given in Table I.

During the normal Period, I, a, the rabbits remained constant in weight and each showed a plus nitrogen and a plus phosphorus balance. The same is to be noted in Periods I, b, and I, c, where an extra amount of phosphorus in the inorganic and in the organic form respectively was added.

These three periods indicate that when there is a sufficiency of phosphorus in the food for the needs of the body, the addition of more phosphorus, whether in the form of inorganic or organic, does not influence the results in any conclusive manner, *e. g.*, there is an apparent increase in the digestibility of the protein when organic phosphorus is fed to the black rabbit, whereas the reverse is apparently true in the case of the white rabbit. From the amount of phosphorus excreted in the feces, the figures show that the phosphorus is not so well utilized by the body as is the nitrogen. Whereas the nitrogen is digested to the extent of 71.7 to 85.6 per cent., the phosphorus is only absorbed from 55 to 83 per cent., thus confirming the results of Zadik[2] and others.

In the normal period, I, a, the ratio N : P_2O_5 in food and feces would indicate that whereas in the food there is twice as much nitrogen as phosphoric acid, the relative amounts in the feces are practically the same, indicating that the nitrogen has been absorbed to a greater degree from the intestinal canal. In the periods, I, b and c, owing to the added amounts of phos-

[1] The inorganic phosphorus in solution was given *per os* daily.

[2] *Arch. f. d. ges. Physiol.*, lxxvii, p. 1, 1899.

phorus, this fact is not brought out. Rather is there an indication that relatively more phosphoric acid has been excreted in the feces.

EXPERIMENT II, a, b.

During this series of observations the food was as free from phosphoric acid as it was possible to obtain it. The basal nitrogenous food used was edestin, obtained from hemp seed and containing over 15 per cent. nitrogen and less than 0.5 per cent. of phosphoric acid. In Period II, a, organic phosphorus was added to the phosphorus-poor food, whereas in II, b, the phosphorus was supplied in the inorganic form.

Several facts make the results obtained during Period II, a, hard to explain. In the first place, the food is a highly artificial one; next, as will be seen, this period follows close on to Period I, c, where the black and the white rabbit ingested 3.891 and 5.096 grams nitrogen and 3.188 and 3.851 grams phosphoric acid respectively. During Period II, a, they not only fell from a high plane of phosphorus-metabolism to a comparatively low one, *i. e.*, from an ingestion of 3.188 to 1.740 grams of phosphoric acid for the black rabbit and from 3.851 to 1.895 grams of phosphoric acid in case of the white rabbit, but the normal amount of nitrogen ingested in Period I, c, was replaced by a much higher nitrogen intake in Period II, a, *i. e.*, the black rabbit ingested 1.909 (5.800–3.891) grams of nitrogen and the white rabbit 1.407 (7.503–5.096) grams of nitrogen more than during the preceding period. It is therefore not surprising that both rabbits give a considerable minus phosphoric acid balance, while at the same time giving a very slight plus nitrogen balance. The large amount of ingested nitrogen stimulated the metabolic processes to such an extent as to produce an abnormal nitrogen and phosphoric acid excretion. That the loss of phosphoric acid is to a great extent due to the too abundant supply of nitrogen seemed apparent when this same experiment was repeated with the black rabbit at a later date. In this latter case the nitrogen ingested was 4.510 grams and the phosphoric acid was 1.555 grams, *i. e.*, more nearly corresponding to the normal period. Under these circumstances a minus nitrogen balance was obtained and a slightly plus phosphoric acid balance.

The rabbits remained practically constant in weight during each period.

In Period II, b, where the nitrogen ingested was more nearly normal, we find that the influence of the inorganic phosphorus has produced not only a minus phosphorus balance but a minus nitrogen balance in the case of both rabbits.

The per cent. of the absorbed nitrogen retained in the body is much larger when organic phosphorus is fed than in the case where inorganic phosphorus is given. Although a very slight amount of nitrogen was ingested in II, a, the amount retained was extremely slight, indicating, as Zadik[1] showed, that the nitrogen balance bears no necessary relation to the amount of nitrogen fed.

In Period II, b, the black rabbit appears to have excreted 0.06 gram of inorganic phosphoric acid in the feces. She ingested 0.623 gram in the food and was given 1.145 grams as added inorganic phosphoric acid, whereas the phosphoric acid in the feces amounted to 0.931 gram. There is a minus phosphoric acid balance, however, of 0.284 gram. If this be assumed to have come from the phosphorus of the body tissue, there is then evidence of an excretion of only 0.06 gram of inorganic phosphorus in the feces. This amount is, however, too small for any conclusion to be drawn, especially as this is the only case which cannot be otherwise explained.

As will be seen, this was a highly artificial period and yet the organic phosphorus has certainly been of more benefit in aiding the digestion of the nitrogen than the inorganic phosphorus. In the column marked "Per cent. of Nitrogen Digested," the inorganic phosphorus period shows a digestion of 67.6 per cent. and 87.4 per cent. of the protein for the black and white rabbits respectively, whereas when organic phosphorus was fed the per cent. of nitrogen digested was increased to 76 and 93 per cent. respectively.

During these periods the $N : P_2O_5$ ratio is approximately the same in the urine as in the food, showing that the nitrogen absorption did not proceed to any greater extent than the phosphorus absorption.

[1] *Loc. cit.*

EXPERIMENT III, a, b, c.

During the third series of the experiments the object was to determine whether inorganic phosphorus could replace the organic phosphorus of bran. For this purpose, during Period III, a, normal bran constituted the basal nitrogen and phosphorus food. In III, b, instead of using normal bran, the rabbits were fed extracted bran, *i. e.*, bran deprived of most of its phosphorus by extraction with 0.2 per cent. hydrochloric acid, the necessary amount of phosphorus being supplied *per os* in the form of inorganic salt as in the previous periods. In III, c, the same bran as in III, b, was used, the organic phosphorus being that obtained from bran by extraction with 0.2 per cent. hydrochloric acid and precipitation with a large volume of alcohol.

In Girard and Lindet's work,[1] the former showed that when bran is ingested it is excreted almost entirely unchanged, *i. e.*, over 90 per cent. of the nitrogen is undigested. On the other hand, the solubility of the mineral constituents amounts to 75 per cent. Not only was 75 per cent. of the ash removed from the bran by the digestive juices, but the remaining ash contained no phosphorus.

The results in feeding bran show that the nitrogen is very poorly absorbed, there being a minus nitrogen balance in five out of the six cases. As regards the phosphorus balance, however, we find that only when the rabbits are fed inorganic phosphorus is there any decided minus phosphorus balance. When the rabbits are fed organic phosphorus, the phosphorus balance is a large positive one. The influence of the phosphorus salt on the nitrogen digested is more beneficial in the case of organic than in the case of inorganic phosphorus feeding, both rabbits behaving in a similar manner.

The ratio of nitrogen to phosphoric acid in food, feces, and urine is interesting as indicating a possible excretion of organic phosphorus in the feces, for whereas the organic N : P_2O_5 ratio in the food is over 2, in the feces it is only 0.6. Steinitz[2] called attention to the possible presence of lecithin in the feces in a similar case, and Bergmann[3] suggested that organic phos-

[1] *Le Froment et sa Mouture*, Paris, 1903.

[2] *Loc. cit.*

[3] *Loc. cit.*

phorus may be excreted through the intestinal tract instead of through the kidneys. The organism seems incapable of dissolving and absorbing very large amounts of phosphorus-rich proteids and, therefore, the $N : P_2O_5$ ratio in the feces is almost always smaller than that in the food and urine. In like manner the high $N : P_2O_5$ ratio in the urine is due to the fact that the nitrogen absorption is greater than the phosphorus absorption.

The per cent. of phosphorus absorbed which was retained in the body appears to be much greater under the influence of organic phosphorus than of inorganic phosphorus. Under the influence of the inorganic phosphorus, not only was all the phosphorus excreted but an additional amount of phosphorus was abstracted from the body tissue. On the other hand, the organic phosphorus produced an actual gain of phosphorus to the extent of 16.4 to 37.7 per cent., of the amount absorbed.

In III, b, the amount of phosphoric acid in the food was 0.715 and 0.766 gram, respectively, in the case of both rabbits. The added inorganic phosphoric acid was 1.491 and 1.813 grams, whereas the phosphoric acid in the feces was 1.291 and 1.316 grams respectively, or appreciably greater in both cases than that which was present in the food. This would seem to indicate that some of the added inorganic phosphorus was excreted in the feces as Paton [1] and Meyer [2] suggest in experiments with dogs. When, however, it is considered that there was a minus phosphoric acid balance of 0.775 and 0.819 gram respectively, it may be fairly conjectured that the excess of phosphoric acid in the feces did not come from the added inorganic phosphoric acid as such, but rather came from catabolic processes. This is in agreement with Bergmann [3] that no inorganic phosphorus is excreted by the intestines.

EXPERIMENTS WITH THE DOG.

Owing to limited space our work was confined to one dog, a bitch of eight kilograms being selected. One of the latest cages, the design of Professor L. B. Mendel, of the Sheffield Scientific School, Yale University, was employed. The dog was

[1] *Journ. of Physiol.*, xxv, p. 212, 1900.
[2] *Zeitschr. f. physiol. Chem.*, xliii, p. 1, 1904.
[3] *Loc. cit.*

catheterized at the beginning and end of each period, the feces separated by lampblack and the usual precautions which the experiment demanded were employed.

The experiments were divided into the two following general periods [1]:

IV. (a) Normal food period: cracker-dust-starch-lard mixture, dog biscuit, chopped meat.

(b) Normal food period: cracker-dust-starch-lard mixture, dog biscuit, chopped meat, plus inorganic phosphates.

(c) Normal food period: cracker-dust-starch-lard mixture, dog biscuit, chopped meat, plus organic phosphorus.

V. (a) Phosphorus-free food: cracker-dust-starch-lard mixture, egg albumin, plus inorganic phosphates.

(b) Phosphorus-free food: cracker-dust-starch-lard mixture, egg albumin, plus organic phosphorus.

After several days' confinement in order to bring the dog into nitrogenous equilibrium and to accustom the animal to the changed environment, the experiment was begun on January 17th. The following five days comprise the normal period. During this period the diet consisted of 40 grams of meat, 43 grams of dog biscuit, and 90 grams of cracker-dust-lard-starch mixture per day. The total intake of nitrogen during this period was 15.986 grams and of phosphoric acid, 4.50 grams. The food was heartily eaten. During the next five days in Period IV, b, to the above food were added 3.98 grams of phosphoric acid in the form of disodium phosphate. In Period IV, c, 4.32 grams of organic phosphorus were added to the normal diet. This organic phosphorus was in the form of calcium-magnesium-potassium salt of anhydrooxymethylenediphosphoric acid. Table II, Experiment IV, shows the results of these experiments.

During the normal period the dog gained in weight 0.3 kilogram and accordingly there is a plus nitrogen balance of 2.227 grams (equal to 0.44 per diem) and a plus phosphoric acid balance of 1.117 grams (equal to 0.22 per diem). These figures are considerably lowered in the inorganic period, being plus 0.261 in the case of both nitrogen and phosphoric acid. The added inorganic phosphorus is not only not retained by the

[1] During each period, 10 grams of salt mixture were given together with the food.

body to any extent but it seems to exert an unduly stimulating effect upon the metabolism, as seen in the reduced nitrogen and phosphoric acid plus balances. We note that the per cent. of phosphoric acid in the feces during this period is the lowest of the three, viz., 23.8 per cent. This does not mean that a larger per cent. of phosphorus has been metabolized but is a simple illustration of the osmotic and absorptive power of the intestinal cells and of the power of the kidneys to excrete soluble compounds that are not available or necessary to the physiological needs of the body. There is a marked rise in the plus nitrogen and plus phosphoric acid balances during the organic period, the excess of phosphoric acid in this case being partly stored up in the body and also exerting a favorable effect on the nitrogen metabolism. The percentages of nitrogen digested during these three periods are practically identical. The per cent. of absorbed nitrogen retained, while greatest during the normal period, due largely to the fact that the animal gained in weight, is markedly lowered during the inorganic phosphoric acid feeding and is in turn increased during the organic phosphorus feeding.

The N : P_2O_5 ratio is higher in the urine than in the food, showing as Steinitz[1] has pointed out that the nitrogen excretion exceeds that of the phosphoric acid.

The object of the next set of experiments was to feed a phosphoric-acid-free food, or one containing the least possible amount of phosphoric acid and to add to that food inorganic and organic phosphorus respectively in amounts sufficient to make the total phosphoric acid ingested equivalent to that fed in the normal food. The general criticism of experiments of this kind is the fact that the food fed is an artificial food, but when the same food is fed, although an unnatural one, the results are comparable at least.

The food during these periods was fed per diem in the following amounts:

Cracker-dust-lard-starch mixture, 100 grams; egg albumin, 21 grams; to which was added 3.22 grams of disodium phosphate or its equivalent in organic form.

The total nitrogen and phosphoric acid ingested during the five days was approximately 17 grams and 4.2 grams respec-

[1] *Loc. cit.*

tively, while during the normal period the dog received 15.98 grams of nitrogen and 4.5 grams of phosphoric acid. The results are collected in Table II, Experiment V.

In the inorganic period, V, a, the added phosphorus was in the form of disodium phosphate. The third period, V, c, recorded above, is an exact repetition of V, a, and the two agree remarkably well, both the minus nitrogen and the minus phosphoric acid balances being large and almost equal. In the case of the organic phosphorus period, V, b, where to the phosphorus-free food egg-yolk was added, the minus nitrogen balance is greatly reduced and instead of a minus phosphoric acid balance of one gram or more a plus phosphoric acid balance of 0.2 gram is noted. The fact that egg-yolk was fed during the organic period, which necessitated a cutting down of the amount of egg-albumin fed to 10 grams per diem, may largely account for the decrease of the minus nitrogen balance, but the plus phosphoric acid balance can only be accredited to the better assimilation of the organic phosphorus. The per cent. of nitrogen digested, as seen in the table, is highest during the organic period. The feces during this period contain only 6 grams of nitrogen, while in the inorganic periods the amount is much larger, due to the indigestibility of the egg-albumin, which could be detected macroscopically. The amount of phosphoric acid in the urine in the organic period is accordingly less than that found in the urine of the inorganic periods. This again bears out our statement that the amount of phosphoric acid in the urine is no evidence of the amount metabolized but merely shows an absorption and rapid elimination of the soluble inorganic phosphorus. The two last columns, showing the per cent. of absorbed nitrogen and phosphoric acid retained are decidedly in favor of the organic phosphorus or egg-yolk period. During each of the three periods the animal showed a slight loss of weight, although nitrogen was fed in the foods in amounts equal to or exceeding that present in the normal period. The loss of weight during this feeding is explained by the inability of the body to digest and assimilate artificial or unnatural foods to an extent equal to that of natural foods.

That egg-albumin is a poor food has been demonstrated by

cent f N gested	Per cent. of Total P_2O_5 in Feces	Ratio, N : P_2O_5 in			$\frac{\text{Urine N}}{\text{Feces N}}$	$\frac{\text{Urine} P_2O_5}{\text{Feces} P_2O_5}$	Per cent. of absorbed N and P_2O_5 Retained	
		Food	Feces	Urine				
1 7	45 0	1 8	1.1	4 6	2.5	0.6	3.0	50.3
0 4	38 6	2 0	1.0	3 0	3 7	1.3	9.0	20.2
4 8	30.5	2.0	1.0	2 8	3.9	1.4	30.1	36.9
3 8	28 0	0 9	0.8	0 9	2 2	1.9	20 3	25.0
7 2	33 8	1 2	0 8	1 6	2 6	1.4	22.6	30.6
2 6	17 3	1 3	1.3	1.9	4.8	3.4	−0.4	5.5
8 7	31 3	1 4	0.9	1.5	3 0	1.8	18.2	17.2
5 6	28 2	1 2	0 6	1 6	4 9	1 9	16.9	23.4
8 0	23 6	1 3	1.2	1.7	3 2	2 2	9.5	31.8
5 3	63 5	3 3	1 3	2 5	2 9	1 5	3 2	−162.6
2 9	14 8	4 0	1 9	3 6	13 1	7 0	0 0	−20 4
5 5	38 4	2 9	1 8	4 2	3.7	1 5	−13 0	3.9
7 6	52 7	2.7	1 7	3 5	2 4	1 2	−15 8	−29 6
4	12 7	2 5	3 0	2.7	7.3	8 0	−32.3	−16 0
0 7	29 1	2 0	0 6	4 3	9 9	1 5	−2 0	39 5
5 6	59 2	1 8	0 4	4 1	7 3	0 8	−21 9	−10 3
4 6	58 5	2 3	0 6	2 3	4 8	1 3	12 8	−84.7
7 4	51 0	2 5	0 6	2 7	7.1	1 6	−1.8	−64.8
3 3	32 0	2 2	0 8	3 4	7 6	1 8	−0 1	16 4
1 6	27 6	2 2	0 7	4 5	11 3	1 6	−3.4	37.7

cent. N sted	Per cent of Total P_2O_5 in Feces	Ratio, N · P_2O_5 in			$\frac{\text{Urine N}}{\text{Feces N}}$	$\frac{\text{Ur.} P_2O_5}{\text{Fec.} P_2O_5}$	Per cent. of absorbed N and P_2O_5 retained	
		Food	Feces	Urine				
7	32.3	3 6	1 5	6 0	5.5	1.3	16.1	36.6
2	23 8	1 9	1 0	2 3	6.7	3.1	1.8	4.0
1	30 5	2 0	0 9	2.8	5.9	1.9	5.3	16.6
9	20 3	4 0	11 7	2 0	1.0	5.5	−40 1	−40.0
3	25 0	4 0	5 9	3 7	1.8	2 8	−3.5	6.3
21	27 5	4 2	12 2	1 7	0 5	3 4	−80.1	−27.8

Steinitz.[1] It produces vomiting and diarrhœa but can be used as a food for a limited number of days, provided a normal diet is fed occasionally. The N : P_2O_5 ratios give some interesting results both in Series IV and V in the organic period, it being highest in the feces and lowest in the urine. This shows that the organic phosphorus at least favors the absorption of nitrogen from the intestinal canal. Noel Paton[2] claims that in the case of dogs the greater part of the phosphoric acid is not excreted through the kidneys, and Falck[3] found that on injecting disodium phosphate only from 56 to 70 per cent. appeared in the urine. Our results would show that most of the added phosphorus, whether organic or inorganic, is excreted through the kidneys.

CONCLUSIONS.

From the limited number of experiments carried out, evidence points to the following:

(1) The amount of nitrogen retained is generally lowered by the addition of inorganic phosphorus when fed with a normal food. The nitrogen balance is not necessarily negative.

(2) In the case of phosphorus-poor food, the addition of inorganic phosphorus decreases the digestibility of the nitrogen and the nitrogen and phosphorus balances are usually negative.

(3) Organic phosphorus favors nitrogen metabolism and increases the nitrogen and phosphorus retention, especially in the case of the phosphorus-poor food. Organic phosphorus, therefore, is more favorable to nitrogen and phosphorus retention than is inorganic phosphorus. The phosphorus of wheat bran appears to be a most valuable food constituent.

(4) The nitrogen and phosphorus balances do not run parallel in all cases; the general tendency is in that direction.

(5) In no case has there been any retention of the added phosphorus, whether fed in the organic or in the inorganic form, when given with a food containing a normal amount of phosphorus.

(6) In no case was organic phosphorus found in the urine

[1] *Loc. cit.*

[2] *Loc. cit.*

[3] *Arch. f. path. Anat. u. Physiol.*, liv, p. 173, 1872.

even when an extra amount of organic phosphorus was ingested.

(7) The minus nitrogen balances obtained during the bran feeding periods agree with Girard's results on the indigestibility of bran nitrogen and corroborate his work on the absorption of bran phosphorus.

The authors wish to thank Dr. H. W. Wiley for valuable suggestions and criticism.

NITROGENOUS METABOLISM AS AFFECTED BY DIET AND BY ALKALINE DIURETICS.

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(Received for publication, June 17, 1906.)

Folin's recent reports[2] of elaborate analyses of normal urines under various dietary conditions led us to carry out the experiments described in this and in a following paper. Our reasons for studying the effect of alkaline diuretics will be explained later in the proper connection.

The subjects of the experiments reported here were three normal men, two of whom were medical students and the other the writer. All three had good muscular development, and merely an ordinary amount of fatty tissue. All were in perfect health during the experiments except that E had a mild pharyngitis for a day or two. In reporting the body weight a deduction was made for the weight of clothing in each case. Students E and L were undergoing considerable mental exertion (though not unusual exertion) but very moderate muscular exertion. H. D. H., however, had considerable muscular exercise and moderate mental exertion.

The diets were purin-free except for Days 13–16 in the case of H. D. H. (Table I). Purin-containing beverages and also potatoes were strictly abstained from. The nitrogen content and the fuel value of the food were calculated from Atwater's tables.[3] The nitrogen waste was calculated approximately, account being taken of the varying degree of digestibility of

[1] H. M. Hanna Research Fellow, Western Reserve University.

[2] Folin, *Amer. Journ. of Physiol.*, xiii, pp. 45, 66, 1905.

[3] Atwater, "Chemical Composition of American Food Materials," *U. S. Dept. of Agriculture*, Bulletin 28.

different kinds of food, then by deducting this waste from the nitrogen content we secured the figures for the "net nitrogen income." In the case of H. D. H. no account was kept of the fuel value, and it is quite certain that his diets were deficient in calories. Each of the other men averaged about 3000 calories.

The *low* diet was a vegetable diet to which a little milk and cream and considerable butter were added. Cornstarch, tapioca, and sugar were used in as large amounts as possible. Both E. and L. used much more fruit than did H. D. H. What is termed the *standard* diet contained about 15 grams of nitrogen and consisted of a considerable quantity of eggs and milk in addition to the vegetable diet, with the change of increasing the amount of bread taken and lessening the amount of purely starchy food. The *heavy* diet contained about 23 grams of nitrogen and was of the same nature as the standard diet. The *meat* diet contained 390 grams of very lean chopped beefsteak, besides milk, bread, butter, and dates.

The following methods [1] of analysis were used. Kjeldahl's method was used for estimating total nitrogen, Folin's method was used for urea, 5 c.c. of urine being taken for each of these estimations, while the ammonia was determined by Folin's method. In making ammonia estimations the air-stream was continued for at least three hours and we know that the results are accurate for we have tested our apparatus a number of times with pure solutions of ammonium salts.

Purins were estimated in all cases but are not reported in the tables below because they are the subject of special consideration in a separate article. An attempt was made to estimate creatinin in two experiments, but the results were not considered reliable. In the case of L., by using a modification of Folin's method similar to Nesslerizing, our estimations showed a practically constant creatinin excretion. As we do not feel sure of the legitimacy of our modification, we refrain from publication.

The complete tables of our results (I–III) will be given first, and later smaller tables of data selected for special purposes will be given in connection with the discussion.

[1] For a description of methods see Folin, *loc. cit.*

TABLE I. H. D. H.

Urine Number	Date 1905–06	Volume of Urine. C. c.	Nitrogen in Food. Grams.	Net Nitrogen Income Grams.	Nitrogen in Urine. Grams.	Urea Nitrogen. Grams.	Ammonia Nitrogen. Grams.	In Per Cent. of Total Nitrogen. Urea N.	In Per Cent. of Total Nitrogen. Ammonia N.	Remarks.
1	Dec. 27	1300	5.15	4.90	7 790	—	—	—	—	Low diet. Weight Dec. 27, 63 5 Kg.
2	" 28	1770	4.13	3.70	6.938	5.227	0.564	75.3	8.1	
3	" 29	1030	3.54	3.18	5.711	4.141	0.472	72.5	8.2	
4	" 30	1465	3.48	3.12	6.922	5.183	0.420	74.8	6.0	Weight Dec.30, 62.5 Kg.
5	" 31	1095	3.51	3.13	5.943	4.735	0.123	79.6	2.0	Sodium citrate administered sufficient to render urine alkaline.
6	Jan. 1	935	—	—	6.985	5.474	0.172	78.3	2.5	Citrate continued.
7	" 3	965	15 04	14.33	9.605	7.795	0 665	81.1	6.9	No urine saved Jan. 2. Standard diet begun Jan. 2.
8	" 4	1025	15.28	14.60	10.418	8.564	0 764	82.2	7.3	
9	" 5	920	15.18	14.52	12.790	10.652	0.885	83.2	6.9	Weight Jan. 5, 62.2 Kg.
10	" 6	830	15.23	14.57	11.132	8.715	0.903	78.3	8.1	
11	" 7	880	15.12	14.38	11.881	9.580	0.548	80.6	4.6	Sodium citrate in 5 gm. doses three times a day.
12	" 8	830	15.12	14.41	10.876	8.710	0.438	81.0	4.0	Citrate continued.
13	" 9	1155	16.54	15.71	13.421	11.109	0.663	82.7	4.9	Meat diet begun Jan. 9.
14	" 10	910	16.44	15.62	14.243	11.746	0.694	82.4	4.9	Weight Jan.10, 61.4 Kg.
15	" 11	770	16.44	15.62	14.898	12 548	0.760	84.2	6.0	
16	" 12	825	16.44	15.62	15.015	—	0.725	—	4.8	Weight Jan.13, 61.6 Kg.

TABLE II. STUDENT L.

Urine Number.	Date. 1906.	Volume of Urine. C. c.	Nitrogen in Food. Grams.	Net Nitrogen Income. Grams.	Nitrogen in Urine. Grams.	Urea Nitrogen. Grams.	Ammonia Nitrogen. Grams.	In Per Cent. of Total Nitrogen		Remarks.
								Urea N.	Ammonia N.	
1	Feb. 7	1122	4.62	4.34	9.990	8.514	0.503	85.2	5.0	Low diet. Weight Feb. 7, 56.4 Kg.
2	" 8	995	5.81	5.47	7.271	6.146	0.512	84.5	7.0	
3	" 9	1200	5.87	5.52	6.350	5.033	0.511	79.2	8.0	
4	" 10	1318	5.43	5.05	5.904	4.074	0.649	69.0	11.0	
5	" 11	810	5.50	5.11	3.856	2.735	0.304	70.9	7.8	Weight Feb. 11, 56 Kg.
6	" 12	1850	5.10	4.74	6.164	4.506	0.466	73.1	7.5	
7	" 13	1200	6.26	5.83	5.174	3.864	0.188	74.6	3.6	Sodium citrate administered 5 gm. three times a day. Urine alkaline.
8	" 14	960	5.40	5.02	5.053	3.677	0.193	72.8	3.8	Weight Feb. 14, 57 Kg. Fuel value of food for low-diet period averaged 3018 calories.
9	" 15	1375	23.31	22.15	7.469	5.867	0.446	78.5	5.9	Heavy diet, begun Feb. 15.
10	" 16	1375	23.18	22.03	12.050	10.287	0.801	85.3	6.6	
11	" 17	1255	23.65	22.47	14.829	12.510	1.054	84.3	7.1	
12	" 18	1085	23.15	22.00	15.858	13.677	1.118	86.2	7.0	
13	" 19	975	23.31	22.15	14.278	11.630	1.212	81.4	8.4	
14	" 20	1420	23.39	22.23	14.870	13.033	0.723	87.6	4.8	Weight Feb. 19, 57.3 Kg. Sodium citrate (as above.) Urine alkaline.
15	" 21	1840	23.13	21.98	16.229	14.271	0.330	87.9	2.0	Citrate continued. Urine alkaline.
16	" 22	1285	23.73	22.55	15.615	13.471	0.993	86.2	6.3	Weight Feb. 23, 57.5 Kg. Fuel value of food for heavy diet period averaged 3155 calories.

TABLE III. STUDENT E

Urine Number.	Date 1906	Volume of Urine C c	Nitrogen in Food Grams	Net Nitrogen Income Grams.	Nitrogen in Urine Grams	Urea Nitrogen Grams	Ammonia Nitrogen Grams	In Per Cent of Total Nitrogen: Urea N	In Per Cent of Total Nitrogen: Ammonia N.	Remarks.
1	April 20	602	5 10	5 01	5 718		0 303		5 2	Low diet. First 3 days' urine not examined. Weight Apr. 20, 71.2 Kg.
2	" 30	1510	3 25	2 91	7 292	1 651	0 380	63 7	5 2	Weight Apr. 30, 70 3 Kg.
3	May 1	750	5 67	5 20	4 725		0 395		8 3	Had mild pharyngitis May 1
4	" 2	1000	6 01	5 51	5 096	3 276	0 311	61 2	6 1	
5	" 3	1165	5 60	5 14	6 752	1 697	0 130	69 5	1 9	NaHCO₃ administration, sufficient to keep urine alkaline
6	" 4	1010	5 10	4 91	5 628	3 960	0 085	70 3	1 5	NaHCO₃ continued. Fuel value of food in low-diet period averaged 3071 calories
7	" 7	760	15 17	14 36	8 363		0 161		5 5	Standard diet, begun May 7. Weight, 71.1 Kg.
8	" 8	1000	14 30	13 56	10 696	8 862	0 392	82 8	3 6	
9	" 9	1065	14 93	14 08	10 881	8 717	0 567	80 0	5 2	
10	" 10	1023	14 82	13 98	8 150		0 189		2 2	
11	" 12	870	15 30	14 19	10 913		0 331		3 0	NaHCO₃ (as above). Urine not saved May 11 (12 hours).
12	" 13	780	15 56	14 66	11 619		0 521		4 5	Weight May 12, 71 7 Kg.
13	" 15	920	14 80	14 01	13 885	10 626	0 705	76 5	5 0	Urine not saved May 14.
14	" 16	925	14 80	13 07	12 173	9 812	0 202	80 8	1 6	
15	" 17	1120	15 02	14 13	11 885		0 395		3 3	Sodium citrate administered in 5 gm doses (total 20 25 gms.). Urine alkaline.
16	" 18	765	14 89	14 09	11 207		0 677		6 0	Urine still alkaline
17	" 19	880	15 19	14 30	11 581		0 660		5 7	
18	" 20	830	14 89	14 09	13 119		0 811		6 1	
19	" 21	805	14 71	13 86	13 382		0 862		6 1	
20	" 22	815	15 53	14 61	12 371		0 799		6 1	
21	" 23	1080	15.60	14 69	12 338		0 212		1 7	
22	" 24	1305	15 07	14 20	12 460		0 115		0 9	Sodium citrate (as above). Urine alkaline.
23	" 25	1410	14 88	14 07	10 738		0 112		1 3	Citrate continued. Urine alkaline.
24	" 28	1000	15 11	14 19	14 420		0 806		5 5	Citrate continued. Urine alkaline.
25	" 29	922	14 96	14 06	14 022		0 965		6.4	
26	" 30	635	14 59	13 72	11 593		0 686		5.9	2 days' urine not saved. Fuel value of food for this dietary period averaged 3135 calories.

Only a few urea estimations were made in the case of E. (Table III) because we cared merely to study, in so far as urea was concerned, the effect of alkali-producing drugs, having previously become convinced of the correctness of Folin's results as regards the effect of diet on urea excretion.

A few general observations on the tables will be offered first. Neither sodium citrate nor sodium bicarbonate influenced the amount of total nitrogen excretion. They also failed to cause diuresis, the increase of urine volume in the case of Numbers 14 and 15, Table II, and 22 and 23, Table III, being due undoubtedly to increased ingestion of water.

It will be noticed that the sudden change to a low diet caused in each experiment an initial fall in body weight, but this was quickly recovered from in both cases where the fuel value was sufficient (Tables II and III). The weight of these two men increased slightly on the heavier diet. H. D. H. (Table I), however, in whose case the calories were low in amount, lost weight steadily until the meat diet was begun.

The tables show that nitrogenous equilibrium is quickly secured if the nitrogen intake does not drop below 5 grams, as others have also observed. The nitrogen excretion remains fairly constant at 5–6 grams even when the food-nitrogen drops below that amount. Folin had a similar experience, for on a diet containing only one gram of nitrogen the excretion varied for the most part from 3.5 to 5.5 grams. On the standard and on the meat diet an approximate nitrogenous equilibrium was readily obtained; on the heavy diet (Table II), however, equilibrium was not secured, 16 grams being the maximum nitrogen excretion.

The effect of diet on urea and ammonia excretion will be next studied with the aid of the following tables (IV–VII) in which the urine numbers correspond to those in Tables I–III.

The three students, A, B, and C, were kept on a low diet similar in character to that in our experiments for a period of six to seven days, the last day's urine (with A, two days) being examined in order to secure additional evidence in regard to ammonia excretion.

As regards the per cent. of urea nitrogen on low, standard, and heavy diets, our results corroborate Folin's findings, *i. e.*, that

the per cent. of urea nitrogen is less on a low diet, averaging 71.4 per cent. in our series as against 82.2 per cent. on the higher diets.

TABLE IV.

THE EFFECT OF LOW DIET ON UREA AND AMMONIA EXCRETION.

	Urine Number.	Nitrogen in Urine Grams	Urea Nitrogen Grams	Ammonia Nitrogen Grams	In Per Cent of Total Nitrogen	
					Urea N	Ammonia N.
H. D. H.	2	6 938	5 227	0 564	75 3	8 1
(Table I.)	3	5 711	4 141	0 472	72.5	8 2
	4	6 922	5 183	0 420	74 8	6 0
Average					74 2	7 4
L	3	6 350	5 033	0 511	79 2	8 0
(Table II.)	4	5 904	4 074	0 649	69.0	11 0
	5	3 856	2 735	0 304	70 9	7 8
	6	6 164	4 506	0 466	73 1	7 5
Average					73 0	8 6
E	1	5 748		0 303		5 2
(Table III)	2	7 292	4 651	0 380	63 7	5 2
	3	4 725		0 395		8 3
	4	5 096	3 276	0 314	64 2	6 1
Average					63 9	6 2
Average of all					71 4	7 4
Student A		7 75		0.365		4 7
		7 16		0.417		5 8
Student B		5 63		0 336		5.9
Student C		7 76		0 428		5 5
Final Av'rge of all					71 4	6 9

As to the ammonia nitrogen we find practically the same percentage on all diets, although it is a little lower on the meat diet (Table VI). H. D. H. averaged 7.4 per cent. on the low diet and 7.3 per cent. on the standard diet. L. averaged 8.6 per cent (or 7.4 per cent. if the exceptional excretion of Number 4 [11 per cent.] be excluded) on the low and 7.3 per cent. on the heavy diet. E. averaged 6 2 per cent. on the low diet and 5.6 on the standard diet. The general average of all examinations on the low diet is 6.9 per cent., and of all on the higher diets is 6.1 per cent. There is, therefore, but one interpretation of

our results possible, namely, that with normal varied diets the ammonia excretion is proportional in amount to the total nitrogen, and consequently the percentage of the total nitrogen present in the urine as ammonia is practically constant.

TABLE V.

THE EFFECT OF STANDARD DIET ON UREA AND AMMONIA EXCRETION.

	Urine Number.	Nitrogen in Urine. Grams.	Urea Nitrogen. Grams.	Ammonia Nitrogen. Grams.	In Per Cent. of Total Nitrogen.	
					Urea N.	Ammonia N.
H. D. H. (Table I.)	7	9.605	7.795	0.665	81.1	6.9
	8	10.418	8.564	0.764	82.2	7.3
	9	12.790	10.652	0.885	83.2	6.9
	10	11.132	8.715	0.903	78.3	8.1
Average					81.2	7.3
E. (Table III.)	8	10.696	8.862	0.392	82.8	3.6
	9	10.884	8.717	0.567	80.0	5.2
	12	11.619		0.524		4.5
	13	13.885	10.626	0.705	76.5	5.0
	16	11.267		0.677		6.0
	17	11.581		0.660		5.7
	18	13.119		0.841		6.4
	19	13.382		0.862		6.4
	20	12.374		0.799		6.4
	24	14.420		0.806		5.5
	25	14.922		0.965		6.4
	26	11.593		0.686		5.9
Average					79.7	5.6
Average of all					80.5	6.0

Folin drew a different conclusion from his analyses, viz., *that the* per cent. of ammonia is relatively high on a low diet. We must, therefore, make a comparative study of his tables. In the first place, one of his subjects, Dr. H. B. H. (Table VII, p. 108), gave the same relative results as ours, averaging 3.2 per cent. on the low diet and 3.1 per cent. on the ordinary diet. This man was on diets similar in nature to ours. Another case in which Folin found a low per cent. of ammonia on a low diet (of the same kind as with Dr. H. B. H.) was O. F. (Table VI, p. 106) who averaged 3.7 per cent. Furthermore the vegetarian, Dr. E. v. S. (Table I, p. 70), on his accustomed low diet showed an average ammonia excretion of 6.9 per cent. (excluding the two days on a heavier diet), which is the same as our general

low diet average. Folin's results which are at variance with these appear only in the cases (Tables II–V, pp. 74–80, and VIII–IX, pp. 110–112) where he gave a starch-cream diet containing about 1 gram of nitrogen. In all of these the ammonia nitrogen shows a much higher per cent. on this peculiar low diet than on the standard diet, reaching an average as high as 8.1 per cent., 8.7 per cent., and 10 per cent. with three individuals while on that low diet. It seems quite probable that Folin's results were due to the abnormal character of his low diet. Fortunately for comparison one of the men whom he experimented upon, Dr. H. B. H., tried both the normal, varied, low diet and the starch-cream, low diet (Table VII, p. 108, and III, p. 76); on the former diet, as has been mentioned, the results agree with ours, the per cent. of ammonia nitrogen being the same on the high and low diets, while on the abnormal diet the per cent. was quite high (8.7 per cent.). This indicates that the conditions of Folin's experiments with the starch-cream diet were decidedly exceptional. On a diet containing but 1 gram of nitrogen considerable waste of tissue proteid must be involved, since the nitrogen excretion exceeded the nitrogen income by 1.7 to 4.4 grams (average about 3 grams). Thus a new factor not comparable with physiological conditions would seem to have been introduced.

TABLE VI.

THE EFFECT OF FLESH DIET ON UREA AND AMMONIA EXCRETION.

	Urine Number	Nitrogen in Urine. Grams.	Urea Nitrogen Grams	Ammonia Nitrogen. Grams	In Per Cent. of Total Nitrogen	
					Urea N.	Ammonia N.
H D. H.	13	13 421	11 109	0 663	82 7	4.9
(Table I.)	14	14 243	11 746	0 694	82.4	4.9
	15	14 898	12 548	0 760	84 2	6 0
	16	15 015		0 725		4.8
Average					83 1	5.1

Folin states his conclusions in regard to ammonia as a law, in the following words: "With pronounced diminution in the protein metabolism (as shown by the total nitrogen in the urine), there is usually, but not always, and therefore not necessarily, a decrease in the absolute quantity of ammonia eliminated. A pronounced reduction of the total nitrogen is, however, always accompanied by a relative increase in the

ammonia nitrogen, provided that the food is not such as to yield an alkaline ash."

TABLE VII.

THE EFFECT OF HEAVY DIET ON UREA AND AMMONIA EXCRETIONS.

	Urine Number.	Nitrogen in Urine. Grams.	Urea Nitrogen. Grams.	Ammonia Nitrogen. Grams.	In Per cent of Total Nitrogen.	
					Urea N	Ammonia N.
L.	10	12.050	10.287	0.801	85.3	6.6
(Table II.)	11	14.829	12.510	1.054	84.3	7.1
	12	15.858	13.677	1.118	86.2	7.0
	13	14.278	11.630	1.212	81.4	8.4
Average					84.3	7.3
Average of Tables V-VII.					82.2	6.1

It may be argued that the reason why our results differ from Folin's is that our diets contained considerable "alkaline ash." This is hardly probable because in our series the lowest per cent. of ammonia excretion was not on the diet containing the largest amount of alkaline ash but on the one containing very little (the meat diet); also the amount of alkaline-ash-containing food was very different with our three subjects, H. D. H. taking the least, while L. and E. took much more, yet the ammonia percentages were practically the same. Furthermore, if the alkaline ash is such an important factor on a low diet, the vegetarian, Dr. E. v. S., should have shown a much lower ammonia per cent. than he did (6.9 per cent.).

In the light of our experiments and some of Folin's the following generalization seems warranted: *On all normal diets (containing at least 5 grams of nitrogen) whether consisting mainly of vegetable, or of mixed, or mainly of animal food, the ammonia excreted varies in amount in the same direction with the total nitrogen and the per cent. of the total nitrogen excreted as ammonia nitrogen is generally almost constant for any particular individual.* Of course, we have not determined whether the above would hold true if disturbing factors, such as an increase of mental and muscular exertion, etc., should be introduced.

We now come to the question of the effect of administration of sodium bicarbonate and sodium citrate on urea and ammonia excretion. The following tables (VIII–X) will facilitate the study of this matter.

TABLE VIII.

LOW DIET. EFFECT OF ALKALIES ON AMMONIA AND UREA EXCRETION.

	Urine Number.	Nitrogen in Urine. Grams.	Urea Nitrogen. Grams.	Ammonia Nitrogen. Grams.	In Per Cent. of Total Nitrogen: Urea N.	Ammonia N.	Remarks.
H. D. H. (Table I.)	3	5.71	4.14	0.472	72.5	8.2	
	4	6.92	5.18	0.420	74.9	6.0	
	5	5.94	4.73	0.122	79.6	2.0	Citrate day.
	6	6.98	5.47	0.171	78.3	2.4	Citrate day.
L. (Table II.)	5	3.85	2.73	0.304	70.9	7.8	
	6	6.16	4.50	0.466	73.1	7.5	
	7	5.17	3.86	0.188	74.6	3.6	Citrate day.
E. (Table III.)	2	7.29	4.65	0.380	63.7	5.2	
	4	5.09	3.27	0.314	64.2	6.1	
	5	6.75	4.69	0.130	69.5	1.9	Bicarbonate day.
	6	5.63	3.96	0.085	70.3	1.5	Bicarbonate day.

TABLE IX.

THE STANDARD DIET. EFFECT OF ALKALIES.

	Urine Number.	Nitrogen in Urine. Grams.	Urea Nitrogen. Grams.	Ammonia Nitrogen Grams	In Per Cent. of Total Nitrogen: Urea N.	Ammonia N	Remarks.
H. D. H. (Table I.)	10	11.13	8.71	0.903	78.3	8.1	
	11	11.88	9.58	0.548	80.6	4.6	Citrate day.
	12	10.87	8.71	0.438	81.0	4.0	Citrate day.
E. (Table III.)	13	13.88	10.63	0.705	76.5	5.0	
	14	12.17	9.84	0.202	80.8	1.6	Citrate day.
E. (Table III.)	9	10.88		0.567		5.2	
	10	8.45		0.189		2.2	Bicarbonate day.
E. (Table III.)	19	13.38		0.862		6.4	
	20	12.37		0.799		6.4	
	21	12.34		0.212		1.7	Citrate day.
	22	12.46		0.115		0.9	Citrate day.
	23	10.74		0.142		1.3	Citrate day.

TABLE X.

HEAVY DIET. EFFECT OF ALKALIES.

	Urine Number.	Nitrogen in Urine. Grams.	Urea Nitrogen. Grams.	Ammonia Nitrogen. Grams.	In Per Cent. of Total Nitrogen.		Remarks
					Urea N.	Ammonia N.	
L. (Table II)	12	15.86	13.68	1.12	86.2	7.0	
	13	14.28	11.63	1.21	81.4	8.4	
	14	14.87	13.03	0.723	87.6	4.8	Citrate day.
	15	16.23	14.27	0.330	87.9	2.0	Citrate day.

It will be seen that the effect of the alkalies in decreasing the ammonia excretion was very marked under all conditions of diet, the ammonia being reduced usually to one third of the normal amount. We, of course, expected this result, since other investigators[1] have made the same observation.

We desired, however, to determine whether the urea was correspondingly increased when the ammonia was decreased. The tables show that it was, the increase in per cent. of the total nitrogen appearing as urea nitrogen averaging 2.5, 2.6, 4.25, 4.3, 5.2, and 6.0 in the different alkali periods. The amount of increase corresponded roughly to the amount of ammonia decrease. No comparison can be made of the absolute amounts of urea or ammonia nitrogen since the total nitrogen is so variable.

The explanation for this decrease of ammonia and increase of urea is undoubtedly as follows:[2] Ordinarily the acids formed in the body combine in part with the ammonia of the blood and in such combination much of the ammonia escapes conversion into urea; if, however, the fixed alkali of the blood be increased in amount (as by administration of sodium citrate or bicarbonate) it is used in place of a large part of the ammonia for neutralizing those acids: in the presence of the large quantity of carbon dioxide in blood the ammonia combines with it to form ammonium carbonate and carbamate, which are both readily changed to urea by the liver (and probably other organs), so that the out-

[1] Beckmann-Stadelmann, *Einfluss der Alkalien auf den Stoffwechsel des Menschen.* Stuttgart, 1890.

W. Camerer, Jr., *Zeitschr. f. Biol.*, xliii, p. 39, 1902.

[2] See also Camerer, *loc. cit.*

come is marked decrease of ammonia and some increase of urea in the urine.

We have in a number of instances[1] detected the presence of ammonium carbamate in alkaline urine after the administration of citrate.

The reason why some ammonia always slips through into the urine is, as Camerer[2] suggests, that a fraction of the ammonia-containing blood passes through the kidney before reaching organs that are capable of synthesizing urea.

[1]Macleod and Haskins. This Journal, i, p. 331, 1906.
[2]Camerer, *loc. cit.*

SOME OBSERVATIONS ON THE BEHAVIOR OF THE ENDOGENOUS PURIN EXCRETION IN MAN.

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(Received for publication, June 17, 1906.)

Since the publication, in 1900, of Burian and Schur's first paper on the metabolism of the purins,[2] a considerable amount of interest in this subject has been awakened in the physiological world. The derivation of the urinary purins from two sources—from the food (exogenous) and from the tissues (endogenous)—and the variability in the amount of the endogenous moiety in different individuals but its constancy in any one individual while living under normal conditions and on a purin-free diet, are among the most important points established by these workers. Sivèn,[3] quite independently of Burian and Schur, simultaneously demonstrated the dual origin of the urinary purins and the constancy of the endogenous moiety, and during the past five years these laws, as we may call them, have been confirmed by other workers.

More recently, however, Folin[4] has communicated results which throw doubt on Burian and Schur's statement that the endogenous moiety is constant in amount provided that the diet be purin-free (which of course implies that changes in the amount of purin-free food ingested will be without effect on the purin excretion) and, in view of the fact that this point is of fundamental importance in the study of the whole question of purin metabolism, we have thought it advisable to place on record certain results bearing on the subject which we have obtained during the past year.

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[2] Burian and Schur, *Arch. f. d. ges. Physiol.*, lxxx, p. 241, 1900; lxxxvii, p. 239, 1901; and xciv, p. 273, 1903.

[3] Sivèn, *Skand. Arch. f. Physiol.*, xi, p. 123, 1901.

[4] Folin, *Amer. Journ. of Physiol.*, xiii, pp. 45, 66, and 117, 1905.

In the course of these investigations we have also studied the effect of the administration of citrates, and of alkalies, on the endogenous purin excretion, for, so far as we are aware, no work on this aspect of the question has been recorded since the publication of Burian and Schur's paper.

The present investigations were carried out on three perfectly healthy young men of whom one was one of the writers of this paper (H. D. H.) and the other two, students in this college. A moderate amount of work, mental and physical, was practised during the investigation by all three. The behavior of the urinary nitrogen, urea, and ammonia was investigated by one of us, the results being recorded in a separate paper in this Journal, where detailed accounts of the diet, body weight, etc., will also be found.

CONSIDERATION OF RESULTS.

Experiment I. (TABLE I., H. D. H.)

This observation was divided into three periods; during the first of these, the diet consisted largely of tapioca, bread, milk, etc.; it contained 3.5 grams of nitrogen. During the second period, eggs and milk were freely taken, but no meat or other purin-containing food; total nitrogen, 15 grams. During the third period, meat was taken in place of the eggs and milk of the previous diet: total nitrogen, 16.5 grams. Since Folin has found potatoes to influence the purin excretion, these were excluded from the diet throughout.

About ten hours of laboratory work was performed daily. The urine collected during the first day of the low diet was discarded. The Camerer-Arnstein method was that employed for estimating the purins, the average of the duplicates being taken.

On the last two days of the first two periods, citrate of sodium was taken in five-gram doses two or three times a day. This rendered the urine strongly alkaline to litmus and caused it to effervesce briskly on the addition of acids.

The excretion of purin-nitrogen, exclusive of the citrate days, was fairly constant, the average during the second period (0.146

gram)[1] being slightly higher than that during the first period (0.137 gram).

TABLE I.—H. D. H.—BODY WEIGHT, 61–63 KILOS.

No.	Nature of Diet.	Volume of Urine. C. c.	Excreted per diem in Urine: Nitrogen Grams.	Excreted per diem in Urine: Purin-Nitrogen. Grams.	Purin-Nitrogen. Grams. Average	Remarks.
I.	Starch and cream, containing 3.5 gm. nitrogen.					Body weight 63.5 Kg.
II		1770	6.94	0.128	0.137	
III.		1030	5.71	0.135		
IV		1465	6.92	0.150		
V	*Citrate days.*	1095	5.94	0.176		Citrate of sodium in 5 gm. doses 3–4 times a day.
VI		935	6.98	0.126		
						Day missed.
VII	Eggs, milk, etc., containing 15 gm N. but no purins	965	9.60	0.146	0.146	
VIII		1025	10.42	[0.118]		
IX		920	12.79	0.151		
X		830	11.13	0.142		
XI	*Citrate days.*	880	11.88	0.309		Citrate of sodium in 5 gm doses 3-4 times a day.
XII		830	10.88	0.174		
XIII	Diet included 390 gm. lean steak. Total nitrogen 16.5 grams.	1155	13.42	0.186	0.247	
XIV		910	14.24	0.222		
XV		770	15.90	0.273		
XVI		825	15.01	0.246		Body weight 61.6 Kg.

During the third period, a daily ration of 390 grams of lean steak was taken, but no other purin-yielding food stuff. Allowing that lean steak contains 0.06 per cent. of purin nitrogen (Burian and Schur) and that 50 per cent. of this (0.117 gram) appears in the urine as exogenous purins, we have for the endogenous excretion during this period, 0.130 gram of purin nitrogen.

Regarding the effect of citrate administration, it will be noted that during Period I a distinct increase in purin nitrogen

[1] In this average the purin value for Day VIII is omitted on account of errors in technique.

occurred on the first day that citrate was taken, but a diminution during the second day. The average excretion for the two days was 0.151 gram of purin nitrogen. During Period II the increase, thus induced, was very marked on the first day and it was also very distinct on the second day.

Experiment II. (TABLE II, L.)

Throughout this observation a purin- and potato-free diet was taken. For the first eight days, this contained 5.5 grams of nitrogen and during the next nine days 23.2 grams. The energy value of the diet was carefully maintained at about 3000 *C*.

TABLE II.—L.—AVERAGE BODY WEIGHT, 56.7 KILOS.

No.	Nature of Diet.	Volume of Urine. c. c.	Excreted per diem in Urine. Nitrogen. Grams.	Purin-nitrogen. Grams.		Remarks.
I	Purin-free diet with 5.5 gm. nitrogen	1122	9.99	0.269	averages	W'gt. 56.4Kg.
II		995	7.27	0.152		
III		1200	6.35	0.130		
IV		1318	5.90	[0.097]	0.138	
V		810	3.86	[0.093]		W'gt. 56 Kg.
VI		1850	6.16	0.133		
VII	*Citrate day*	1200	5.17	0.128		Sodium citrate taken till urine *just* alkaline.
VIII		960	5.05	0.131		W'gt. 57 Kg.
IX	Purin-free diet with 23.2 grams nitrogen.	1375	7.47	0.133		
X		1375	12.05	0.120		
XI		1255	14.83	0.128	With XVII 0.126	
XII		1085	15.86	0.125		
XIII		975	14.28	0.123		
XIV	*Citrate days.*	1420	14.87	0.256		Sodium citrate till urine distinctly alkaline.
XV		1840	16.23	0.288		
XVI		1285	15.61	0.248		Urine acid. ammonia normal.
XVII		1230	15.43	0.136		W'gt.57.5 Kg.

Omitting Days IV and V when the results were out of line,[1] it will be seen that break down of body proteid occurred during Period I and retention of proteid during Period II.

The average daily excretion of purin-nitrogen (omitting the first and the citrate days in each period) was, for the first period, 0.138 gram, and for the second, 0.126 gram.

Citrate administration did not cause an increase in purins during Period I—although it affected the ammonia excretion (p. 228)—but it had a very distinct influence on this during Period II. In neither period did it affect the excretion of nitrogen.

Experiment III. (TABLE III., E.)

In this case also a purin- and potato-free diet was taken. For the first nine days of the observation this contained a daily average of 5.2 grams of nitrogen: for the remaining twenty days the average was 15 grams. The energy value was about 3000 *C* throughout. The subject was on diet for three days before the urine was analyzed.

Sodium bicarbonate was taken on the last two days of the low-diet period, and again on the fourth day of the liberal diet. Sodium citrate was taken on the fourteenth and again on the twenty-first, twenty-second, and twenty-third days, *i.e.* during the liberal diet.

In this case estimation was made not only of the total purin-nitrogen but also of the uric acid (Folin and Shaffer's modification of Hopkin's method).

The nitrogen excretion behaved as in the previous cases. Disregarding, for the present, the effect of citrate and alkali, it will be noted that the daily average excretion of total purins was the same on the low as on the liberal diet, viz., 0.174 gram of purin-nitrogen for the low, and 0.168 gram for the liberal diet. This is in accord with Burian and Schur's law. On the other hand, a comparison of the uric acid excretions during these diets reveals a somewhat higher average daily excretion on the liberal than on the low diet, viz., 0.143 gram of uric acid nitrogen for the latter, and 0.154 gram for the former. This difference is,

[1] We have, however, recorded the values obtained on these days, since we cannot account for the irregularity.

TABLE III.—E.—AVERAGE BODY WEIGHT, 71.3 KILOS.

No.	Nature of Diet.	Volume of Urine. C. c.	Excreted per diem in Urine. Nitrogen. Grams.	Purin-Nitrogen. Grams.	Purin-Nitrogen. Grams. Average	Uric Acid Nitrogen. Grams.	Uric Acid Nitrogen. Grams. Average	Remarks.
I	Purin-free	602	5.75	—		0.126		Body weight, 71.2 Kg.
II	diet, con-	1510	7.29	0.174		0.136		
III	taining 4.8	750	4.73	0.172	0.174	0.152	0.143	
IV	Gm. N.	1000	5.10	0.176		0.142		
V	*Sodium*	1165	6.75	0.200	0.202	0.146	0.154	
VI	*bicarbo-nate days*	1010	5.63	0.204		0.163		Urine alkaline.
VII	Purin-free	760	8.63	[0.111]		0.137		Two days missed.
VIII	diet con-	1000	10.70	0.178		0.130	0.138	Body wt. 71.4 Kg. Urine acid.
IX	taining 15 grams N.	1065	10.88	0.174	0.176	0.138		" "
X	*Sodium bicarbo-nate day.*	1023	8.45	0.163		0.141		" "
XI		870	10.91	0.191		0.150		Urine alkaline.
XII		780	11.62	0.189		0.156		½ day missed.
XIII		920	13.88	0.200	0.187	0.207	0.168	B. Wt. 71.7 Kg. Ur. acid
XIV	*Sod. citrate*	925	12.17	0.188		0.174		Urine acid.
XV		1120	11.88	0.191		0.180		One day missed.
XVI		765	11.27	0.151		0.137		Urine acid.
XVII		880	11.58	0.164		0.142		Urine alkaline.
XVIII		830	13.12	0.177	0.168	0.166		" "
XIX		895	13.38	0.178		—	0.154	" acid
XX		845	12.37	—		0.172		" "
XXI	*Sodium*	1080	12.34	0.174		—		" "
XXII	*citrate*	1365	12.46	0.179		0.164		" "
XXIII	*days.*	1410	10.74	0.236	0.208	0.183		Urine alkaline.
XXIV		1000	14.42	0.224		0.173	0.174	" "
XXV		922	14.92	0.228		0.177		" "
XXVI		635	11.59	0.140		0.131		Two days missed. Urine acid. " "

however, only noticeable after the liberal diet had been taken for some time and is so small as to be negligible. It is scarcely allowable to infer anything regarding the behavior of the purin bases, since the only values we have for these are calculated from the difference between the purin and uric acid figures. These values for the periods bracketed in the table are as follows: 0.033, 0.048, 0.038, 0.019, 0.014, and 0.034 grams of purin nitrogen.

The administration of alkali, during both the low and the liberal diets, produced a distinct increase in the total purin and uric acid excretions. During the latter diet, this effect did not appear on the day of taking the alkali but was well marked for four days following this, the urine reacting *acid* towards litmus on these latter days. At any rate, we are not able to explain the high purin excretion on Days XI, XII, and XIII in any other way, there having been no irregularity in diet or other unusual condition to explain it.

Citrate of sodium was taken on Day XIV, during the liberal diet. On the first day, it did not influence the previously high purin excretion but it will be noticed that on the two succeeding days a somewhat smaller amount than normal both of purin and uric acid was excreted. It was given later in the experiment (Days XXI, XXII, XXIII), producing no effect until the third day of its administration but being followed by a high excretion lasting several days.

DISCUSSION OF RESULTS.

The constancy of the purin excretion notwithstanding considerable variations in the diet is clearly demonstrated by our results. It is true that in the case of H. D. H. a slight average increase was obtained during the more liberal diet, but, since the excretions of only three days are included in the average, no weight can be given to so slight a variation. This result, like those of Sivèn,[1] Rockwood,[2] Walker Hall,[3] and others, would therefore corroborate Burian and Schur's conclusion that the endogenous

[1] Sivèn, *loc. cit.*

[2] Rockwood, E. W., *Amer. Journ. of Physiol.*, xii, p. 38, 1904.

[3] Walker Hall, *The Purin Bodies of Food Stuffs*, Manchester, 1902; also, *Brit. Med. Journ.*, Sept. 24, 1904.

moiety is uninfluenced by the nature and amount of the diet, provided that this is purin-free and sufficient in amount [and of proper composition][1] to prevent starvation. Folin has, as remarked above, doubted the truth of this law. In two respects, however, Folin's experiments differ from those of Burian and Schur: firstly, in the method used for analysis,[2] and secondly, in that the low diet in Folin's observations contained very much less nitrogen than did that of Burian and Schur.

It is on account of the first of these points that, in the case of our third observation (on E.), we carried out parallel estimations by the two methods employed by these workers. It will be noted that the average results for uric acid are slightly higher on the liberal than on the low diet (viz., 0.143 gram uric acid nitrogen on the low diet as against 0.154 gram on the liberal diet), but the difference is too small to be considered of any account. We must conclude, therefore, that the *apparent discrepancy between the conclusions of the two groups of workers is due to differences in experimental conditions*, viz., to Folin's low diet containing much less proteid than did that employed by Burian and Schur.

Folin's low diet in the two cases in which he noted a very distinct diminution in uric acid contained an average of about one gram of nitrogen and consisted of starch and cream. Burian and Schur's low diet contained over nine grams of nitrogen and was varied in nature: milk and eggs for four days, and vegetables for four other days. The distinct falls in the excretion of uric acid, observed by Folin, occurred when this starch and cream diet was taken. In two of Folin's experiments in which the low diet was a mixed one (*vide* Tables VI and VII of Folin's paper, pp. 106–109 and also p. 90) the endogenous purins were not diminished, even although there was a fall in the total nitrogen excretion of seven and ten grams respectively.

It seems to us that, in the sense in which it is expressed by

[1] The words in brackets are not included in Burian and Schur's statement of the law.

[2] Folin estimated uric acid alone by his and Schaffer's modification of Hopkin's method,—*Zeitschr. f. physiol. Chem.*, xxxii, p. 552, 1901. Burian and Schur estimated not only uric acid but also total purins and purin bases (*vide* Burian and Schur, *loc. cit.*). The total purin nitrogen results are those from which the authors drew their main conclusions.

them Burian and Schur's law[1] holds good notwithstanding Folin's results, for such an unusual diet as this observer employed can scarcely be considered as normal. The extreme importance of the constancy in endogenous purin excretion under normal conditions lies, we think, largely in the fact that it gives us a rational basis from which further work on this intricate subject can be done, and we consider that Burian and Schur, and subsequent workers, have amply justified this view of the case. We ought to take Burian and Schur's endogenous excretion as our working standard, and if we do so then we must conclude that Folin has succeeded in producing variations in this standard by *unusual conditions of diet* just as we, and others, have done by the administration of drugs, and Burian has done by muscular exercise. There can be no doubt that, at no very distant date, many other conditions will be discovered which influence the excretion. It should not be lost sight of that the two groups of workers were studying the question from different standpoints: Folin to show that with the greatest possible variations in purin-free diet certain changes take place in the composition of the urine; Burian and Schur, to show that with distinct, but not excessive, changes in the purin-free diet the purin excretion is unchanged and could therefore be considered as the normal endogenous excretion for that individual.

The individual variation in the endogenous moiety is very noticeable in our results: for H. D. H. it is 0.138 gram purin-nitrogen, for L., 0.133 gram, and for E., 0.173 gram. It is interesting to note that H. D. H. and L. were much smaller men than E., so that Walker Hall's assertion[2] that the endogenous moiety bears a direct relationship to the body weight would seem to be confirmed by our results. The only worker, so far as we are aware, who has questioned this individual factor in determining the amount of the endogenous purin excretion is Loewi.[3] As

[1] Burian and Schur state this part of their law as follows: "Es ist nur dafür zu sorgen, dass die Nahrung das Nahrungsbedürfnis im ganzen deckt" (*loc. cit.*, p. 303).

[2] Walker Hall, *loc. cit.*

[3] Loewi, *Arch. f. exp. Path. u. Pharm.*, xliv, p. 1; also *Arch. f. d. ges. Physiol.*, lxxxviii p. 296.

pointed out by Burian,[1] however, the observations on which Loewi bases his conclusions are inadequate to prove the point.

The Effect of Citrate Administration.—Citrate of sodium was taken by each of the observed persons at two periods during the observation. In the cases of H. D. H. and L. it was taken during the low, and again during the liberal diet. In the case of E. it was taken at two periods during the liberal diet. The effects of the administration on the endogenous purin excretion may be summarized as follows:

During the low diet, in the case of H. D. H., an increase amounting to 29 per cent. of the previous average excretion was caused on the first day of administration, but a decrease on the second day. In the case of L. no change was produced by the administration while on the low diet. During the liberal diet, the citrate caused a marked increase in all the cases, except on the first occasion on which it was given to E. when the previously high purin excretion, following alkali administration, probably masked its effects. The exact manner of increase in the three remaining observations on the liberal diet was different in each case: in the case of H. D. H., the increase on the first day was about 112 per cent. of the previous average amount, but only about 20 per cent. on the second day. In the case of L. the increase on the first day was about 190 per cent., on the second day about 127 per cent. and on the day after the administration had been stopped there was still an increased excretion amounting to about 96 per cent. In the case of E., there was no distinctly increased purin excretion on the first two days of citrate administration, but on the third day an increase amounting to about 40 per cent. was noted. Two days then elapsed before the urine was again examined, but on the next two days (*i. e.*, third and fourth after discontinuing the drug) an increase amounting to about 34 per cent. was present. These averages are calculated from the total purin values, but it will be seen that those for the uric acid agree closely with them.

A similar increase in the excretion of endogenous purins has been noted to follow the taking of salicylate of soda (Walker

[1] Burian, *ibid*, xciv, p. 273, 1902. See also article "The Metabolism of the Purins" in *Recent Advances in Physiology and Bio-chemistry*, edited by L. Hill, Longmans, Green, & Co., 1906.

Hall[1]) and alcohol (Beebe[2]), and from the literature prior to the date of Burian and Schur's first paper we find that an increased excretion of uric acid has been observed after taking citrate of potash or alkalies (Haig,[3] Gorsky[4]). On the other hand, Klemptner[5] and Burchard[6] record a diminution in uric acid as a result of the taking of citrates.

The undoubted increase in the endogenous purin excretion which our results show to follow citrate administrations might be accounted for in various ways, viz.:

1. By some of the citric acid, before it had become completely oxidized, being used to bring about a synthesis of two urea molecules into uric acid. Thus, Wiener[7] has shown that a synthesis of this nature occurs in birds after they have been given such organic acids as lactic, malonic, tartronic, etc., and he further claims that under certain conditions a similar process may take place in mammals.

2. By the citrate raising the alkalinity of the blood and in some way diminishing the destructive power of the organism towards uric acid.

3. By an increase in the amount of blood circulating through the kidneys, whereby, in terms of Lüthje's[8] and Burian and Schur's[9] hypothesis, a larger fraction than normal of the uric acid in the blood would escape through the kidney filter before being destroyed in the liver, etc.

4. For the sake of completeness, we must add to these the hypothesis of Haig[10] that the increased alkalinity of the blood causes uric acid, stored away in the liver and spleen, to be removed by the urine.

[1] Walker Hall, *Brit. Med. Jour.*, Sept. 1904.

[2] Beebe, S. P., *Amer. Journ. of Physiol.*, xii, p. 13, 1904.

[3] Haig, A., *Journ. of Physiol.*, viii, p. 211, 1887.

[4] Gorsky, Inaugural Dissertation (Russian), St. Petersburg, 1888: from Atwater and Longworthy's *Digest of Metabolism Experiments.*

[5] Klemptner, Inaugural Dissertation, Dorpat, 1889, from Atwater.

[6] Burchard, Inaugural Dissertation, Dorpat, 1889, from Atwater.

[7] Wiener, *Beitr. z. chem. Physiol. und Path.*, ii, p. 42, 1902; also *Ergebnisse der Physiol.*, i, Biochem. Abth.

[8] Lüthje, *Arch. f. Verdauungskrankh.*, ii, p. 36, 1896.

[9] Burian and Schur, *Arch. f. d. ges. Physiol.*, lxxxvii, p. 239, 1901.

[10] Haig, *loc. cit.*

Of these possible causes, we may, with certainty, immediately eliminate the third and fourth. The absence of any constant diuresis on the citrate days eliminates the third possibility, as well as the fact that diuresis produced by other causes has no effect on the twenty-four hours' purin excretion (*e. g.* the, taking of diuretics, the imbibition of large quantities of water, beer,[1] etc.). The persistence of the increase for some days after discontinuing the drug (*cf.* Tables II and III) makes Haig's hypothesis untenable, quite apart from the numerous other known facts which stand against it.

Between the remaining two possibilities there exists this difference that in the one—the synthetic hypothesis—the citric acid acts before it is completely oxidized to carbonate, and in the other that it is first oxidized and so renders the blood and urine more alkaline. To decide between these in our third experiment, we administered sodium bicarbonate instead of citrate, with the result that a similar increase in the purin excretion was obtained, which would point to increased alkalinity as at least one of the causes. Exactly how the increased alkalinity brings about the increased purin excretion remains an open question, and the possibilities are many: thus, it may diminish the destruction of uric acid by the liver, etc., or it may in some way stimulate the practically dormant synthesis from urea which Wiener believes can under certain conditions come into evidence in the mammal, etc.

CONCLUSIONS.

1. The excretion of endogenous purins is not affected by very considerable variations in diet provided that this contains no purins.

2. The endogenous purin excretion of different individuals is variable.

3. The administration of citrate of sodium until the urine reacts alkaline towards litmus causes an increased excretion of endogenous purins which may continue for some days after discontinuing the drug, even although the reaction of the urine has meanwhile returned to acid.

4. Sodium bicarbonate similarly administered also increases the endogenous purin excretion.

[1] Burian and Schur, *loc. cit.*, p. 347.

PRELIMINARY COMMUNICATION OF A METHOD FOR ESTIMATING UREA.

By H. D. HASKINS[1]

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(Received for publication, June 17, 1906.)

It may be thought by some that the devising of more urea methods is hardly in order. In the face of the fact that two good methods have recently come into use, namely the Folin and the Mörner-Folin methods, the writer ventures to offer another which he thinks possesses some advantages.

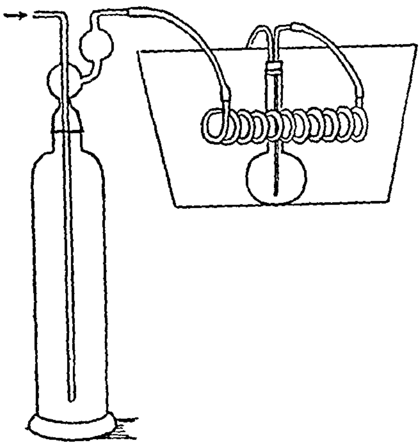
The aim of the new method is great accuracy of estimation under all circumstances, whether the urine examined be normal or pathological, human or animal. It will be noticed that the method is really a modification of the Mörner-Folin[2] method, which is without question the most accurate method in use. As to Folin's method, I wish to take this opportunity for saying that it holds the field as the only reliable method for quick estimation. It, however, is slightly inaccurate, giving a higher estimation than the Mörner-Folin method.

The proposed method is as follows. If the urine is concentrated or contains considerable albumin, dilute it with an equal volume of water. If it is alkaline, acidulate it before measuring the volume of the total urine under examination. With an accurate pipette transfer 5 c.c. of urine to a centrifuge tube, with another add 5 c.c. of a solution of phosphomolybdic acid (10 per cent.) in 10 per cent. hydrochloric acid, cork the tube tightly, and let it stand until the next day (probably six hours would suffice). Shake down thoroughly with a centrifuge, decant the clear supernatant fluid into a small dry flask, and add barium carbonate freely (corking *loosely* after each addition) until effervescence ceases. Then add powdered barium hydroxide (about 0.5 gram), cork tightly, and shake. The precipitate

[1] H. M. Hanna Research Fellow, Western Reserve University.

[2] Mörner, *Skand. Arch. f. Physiol.*, xiv, p. 301, 1903.

becomes deep blue in color and the liquid practically colorless; add more hydroxide if necessary to bring about this condition. Then pour the mixture into a dry centrifuge tube, cork and shake down again, after which decant the clear liquid into a 10 c.c. graduate, and according to its quantity measure 8 c.c. (represents 4 c.c. of urine) or 6 c.c. (represents 3 c.c. of urine) with an accurate pipette.[1] Put this into a 50 c.c. graduated flask.[2] If on adding one drop of alcoholic phenolphthalein solution the fluid becomes pink, neutralize with 10 per cent. hydrochloric acid (a drop at a time), and finally add a little more barium carbonate than will dissolve. The neutral liquid is evaporated to small bulk in a water-bath kept at about 50° C., with the aid of a stream of warm dried air, in the manner to be described.



The flask is fitted with a cork having two holes, in one of which put a short bent glass tube connected with a suction pump; through the other pass a longer tube so that the lower end of it is about 1 centimeter above the surface of the liquid and connect the end of the outer bent part of the tube with a glass worm which lies in the same warm bath that the flask is immersed in (see diagram). This worm is connected in turn with a wash bottle containing concentrated sulphuric acid, so that air sucked into the apparatus is dried by the acid. The flask must be weighted or held by a clamp to keep it well immersed in the bath.

With a good air-stream thirty minutes is usually sufficient to secure evaporation to a volume of about 1.5 c.c. When this volume is attained, disconnect the flask and add 1 gram of powdered barium hydroxide. Put back into the apparatus and run for five minutes.[3] Cool partially and add a mixture of

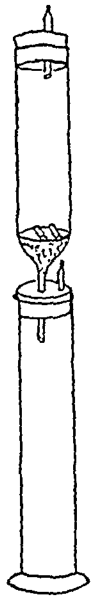
[1] All the pipettes used should be standardized against one another and the 50 c.c. flask.

[2] The flask should have a mouth about 12 mm. in diameter.

[3] There should be enough liquid left to prevent the barium hydroxide from caking.

equal volumes of ether and 99 per cent. alcohol to the mark on the neck. Add an additional 0.5 c.c., to compensate for the displacement of liquid by the barium hydroxide and by the precipitate (make a file mark on the neck for the 50.5 c.c. volume). Stopper tightly and mix. Let the flask stand until the next morning. If necessary to restore the volume to 50.5 c.c. add ether-alcohol. Filter in the special apparatus[1] shown in the accompanying diagram.

This consists of a carbon filter tube fitted onto a 50 c.c. graduate by a rubber stopper, the filter being closed by another rubber stopper, and both stoppers having a small vent tube. The conical bottom of the filter is almost filled with a plug of absorbent cotton rammed down so that it sticks fast but is not too tight, and so that it presents a somewhat even upper surface; two disks of filter paper cut to be slightly larger than the diameter at the surface of the cotton are dropped in and then pressed down and fitted by means of a test-tube held mouth down. The filter disks must be weighted down by several short pieces of glass rod.



Transfer quickly with a pipette about 20 c.c. of the clear ether-alcohol from the flask to the filter, recorking the latter immediately. When it has run through, pour into the filtering apparatus as much more of the ether-alcohol as is possible without stirring up the precipitate. This must be done quickly, and the filtering apparatus must be stoppered at once. As soon as 41–42 c.c. of filtrate have collected, measure 40 c.c. with a pipette and put it into a 300 c.c. flask (round-bottom Jena or an "R" flask). Evaporate at 50° C. with the air-stream apparatus (see above), not quite to dryness. Now add 20 grams of pure crystalline magnesium chloride and 3 c.c. of chemically pure hydrochloric acid and make a Folin's estimation.[2]

In carrying out Folin's method the writer uses at first a trap

[1] The devising of the special apparatus for filtering the ether-alcohol was found to be absolutely necessary to prevent decrease in volume by evaporation. I have found by experiment that the loss during filtration is almost nil. So far as I know such a filtering apparatus has not been described by any one else.

[2] Folin, *Zeitschr. f. physiol. Chem.*, xxxii, p 504, 1901; and xxxvi, p. 333, 1902.

containing some water to act as a valve until enough water has been boiled off so that the magnesium chloride mixture attains the proper concentration, which one can judge after a little experience by the volume of the liquid left in the flask and by the foaming. The flask is then quickly attached to a reflux condenser, which is closed at the top with a cork holding a trap that contains some dilute sulphuric acid. After regulating the flame to secure steady boiling without great foaming, the apparatus requires no attention whatsoever. Boiling for an hour and forty-five minutes is sufficient. In all other particulars the estimation follows Folin's directions. A control of magnesium chloride and the other reagents used must be put through a Folin estimation and the amount of decinormal ammonia (about 0.5 c.c.) obtained must be deducted from every urea estimation. As in the Mörner-Folin method no other correction is called for.

As to the calculation, if 8 c.c. of supernatant fluid was put into the 50 c.c. flask and 40 c.c. of ether-alcohol filtrate was taken, the (corrected) number of cubic centimeters of decinormal ammonia in the distillate must be multiplied by $31\frac{1}{4}$ to give the total cubic centimeters of ammonia corresponding to the urea of 100 c.c. of urine, or multiplying the cubic centimeters of ammonia of the distillate by 0.04375 gives the grams of nitrogen of urea in 100 c.c. of urine, while multiplying by 0.09375[6] gives the grams of urea in 100 c.c.; if 6 c.c. of supernatant fluid was taken, multiply the cubic centimeters of ammonia in the distillate by $41\frac{2}{3}$, or by 0.05833 for nitrogen and by 0.12501 for urea in 100 c.c.

The method has been fully tested with solutions of pure urinary constituents.

(1) Pure urea is accurately estimated, since a solution, 5 c.c. of which gave by Kjeldahl's total nitrogen method 23.4 c.c. of decinormal ammonia in the distillate, gave by this urea method 23.45 c.c. of decinormal ammonia (two estimations).

(2) Ammonium salts do not change the estimation. The urea solution used for (1) after the addition of ammonium sulphate crystals gave for 5 c.c. by this method 23.5 c.c. decinormal ammonia (two estimations).

(3) Kreatinin, hippuric acid, and uric acid could not pos-

sibly affect the estimation, for they are not converted into ammonia by Folin's method (and of these there could only be a trace of hippuric acid remaining in the ether-alcohol solution). Nevertheless an estimation was made with a urea solution (which had been found to give 16.05 c.c. decinormal ammonia for 5 c.c. by Kjeldahl's method) after having added kreatinin (0.2 per cent.), ammonium sulphate, hippuric and uric acids; 5 c.c. gave 16.2 c.c. decinormal ammonia.

(4) Allantoin, which is readily decomposed by Folin's method, and which slightly increases the Mörner-Folin estimation, is eliminated by the method here described. After adding allantoin in excess to the solution used in (3), 5 c.c. gave 15.7 c.c. decinormal ammonia. If the estimations of (3) and (4) be averaged together, the result (15.95 c.c.) is practically identical with the Kjeldahl estimation on the urea alone.

(5) Urochrome is eliminated. A strong solution of urochrome containing a trace of urea gave by this method 1.03 c.c. decinormal ammonia for 5 c.c. while by Folin's method it gave 1.4 c.c. Folin's estimation is apparently increased by urochrome, for a urochrome-urea solution, 5 c.c. of which gave 22.5 c.c. decinormal ammonia, gave after treatment with silver sulphate solution (the silver precipitating urochrome) only 20.85 c.c. decinormal ammonia from the equivalent amount of solution.

The urochrome-urea solutions were obtained by extracting evaporated urine with absolute alcohol. The residue after evaporating the alcohol was dried in the desiccator. On removing from the desiccator, the urochrome took up moisture rapidly from the air and could soon be drained off from the urea crystals. The urea content was proportional to the extent of the deliquescence.

Oxyproteic acid was not obtainable for testing, but it is well known that the treatment with barium and ether-alcohol eliminates it by precipitation.

Thus none of the normal nitrogenous constituents of urine affect the accuracy of the method.

A comparison of estimations on urine by Folin's and Mörner-Folin's methods with those given by the method under consideration is made in the following table, reported as cubic centimeters of decinormal ammonia from 5 c.c. of urine.

COMPARATIVE TABLE OF ESTIMATES.

Urine Number.	Folin's Method. c.c.	Mörner-Folin Method. c.c.	New Method c.c.	Remarks.
1	12.14	16.1	16.37	Diabetic urine.
2	12.6	11.65	11.4	Phosphorus poisoning in a dog.
3	22.66	21.4	21.1	Much bile in the urine.
4	23.94	22.9	22.5	Phenol poisoning.
5	27.8	25.6	25.62	Acute nephritis. About 0.275 per cent. albumin.
6	29.84	27.5	27.36	On standard diet.
7	14.9	14.2	14.45	On low diet.
8	28.85	27.54	26.66	On standard diet.
9	52.0	50.3	49.9	On meat diet.
10	10.56		10.4	
11	14.36		13.33	
12	20.26		20.0	
13	41.35		40.4	
14	40.3		38.8	
15	38.5		37.5	
16	34.35		33.3	
17	46.1		44.5	

Urines 6–17 were normal urines collected for the metabolism experiment recorded in a separate article in this Journal (see Table I., p. 219). As to the result on Urine 1, it is to be recalled that Folin's method is admittedly very inaccurate when sugar is present in the urine.[1]

The ammonia in the urine was estimated by Folin's method, the air-stream being kept up for at least three hours. It will be observed that the Mörner-Folin and the new method give practically identical results, while Folin's method gives a little higher result than the others in every case (except 1).

Since the results of the new method agree so closely with those by the Mörner-Folin method, it seems reasonable to conclude that the former method is as accurate for urine as the latter. What then are the supposed advantages over the Mörner-Folin method? In the first place it is a method in which aliquot parts are taken, obviating all the uncertainty that exists when precipitates have to be washed. Further, it should be more efficient than the Mörner method in eliminating nitrogenous

[1] Mörner, *loc. cit.*; Folin, *Amer. Journ. of Physiol.*, xiii, p. 46, 1905.

bodies other than urea, first, because of the use of phosphomolybdic acid (it may well turn out that with certain pathological urines this method is distinctly more accurate than the others because of the use of this reagent); second, because of the greater concentration of the ether-alcohol than in Mörner estimations, for in the latter almost 10 per cent. of water is present (5 c.c. urine and 5 c.c. barium mixture with 100 c.c. ether-alcohol) while only 2–3 per cent. of water is present in our estimations; also the increase of the proportion of ether (50 per cent. as against 33 per cent. by Mörner's method) is doubtless more effective in throwing some constituents of the urine out of solution: for instance, allantoin is insoluble in ether and absolute alcohol, and we have seen that the new method eliminates it, whereas by the Mörner-Folin method 4.8–7 per cent. of the allantoin enters into the estimation.[1] More hippuric acid is removed by this new method than by any other. Since the reagents used for Folin's estimation, however, do not decompose hippuric acid, this fact is not of practical importance. The use of magnesium oxide is avoided, which is desirable since in the Mörner method this substance can decompose some of the urea if the evaporation temperature be not kept low enough.

The method prevents at least the following nitrogenous bodies from being included in the urea estimation: ammonia, kreatinin, allantoin, hippuric acid, uric acid and other purins, oxyproteic acid, urochrome and some other coloring matters, proteids, and amido acids.

With diabetic urine it is as accurate as the Mörner-Folin method.

The method could undoubtedly be modified for urea estimations in blood.

The cost of reagents for the estimation is less than for the Mörner-Folin method.

Phosphotungstic acid[2] must not be substituted for phosphomolybdic acid, since most samples of the former precipitate urea and some do not precipitate the ammonia completely.

It is the author's intention to test the method more fully in the near future.

[1] Mörner, *loc. cit.*

[2] This is used in the Schöndorff method.

THE DIGESTIBILITY AND UTILIZATION OF SOME POLYSACCHARIDE CARBOHYDRATES DERIVED FROM LICHENS AND MARINE ALGÆ.

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(Received for publication, July 19, 1906.)

During the last few years increased attention has been directed to the employment of a number of peculiar food materials hitherto restricted in use to a few countries. The most familiar of the products are furnished by the algæ and lichens, while some underground storage organs of flowering plants afford the unusual compounds of the inulin type. A recent publication of the Bureau of Fisheries[1] has pointed out that while marine plants are extensively utilized in France, Ireland, Scotland, and other European countries, in the East Indies, in China, and elsewhere, in no other country are such products relatively and actually so important or utilized in such a large variety of ways as in Japan. The products enter extensively into the dietary of the Japanese; and since all of the important preparations can be made from plants easily available in the United States, it has lately been suggested that they ought to find a ready use in dietetic ways in this country.[2] This refers especially to several groups of plant products characterized by their content of types of carbohydrates differing notably from those which constitute the staple nutrients of our diet.

Carrageen, or Irish moss, dulse, laver, and vegetable gelatin, vegetable isinglass or agar-agar, are the most familiar seaweed preparations. Iceland moss represents the lichen products; while the familiar inulin-yielding plants are the edible Jerusalem artichoke (*Helianthus tuberosus*) and the Japanese *Stachys*

[1] H. M. Smith, "The Seaweed Industries of Japan," *Bull. of the Bureau of Fisheries*, xxiv, p. 133, 1904. For the edible algæ and lichens used by the Hawaiians, *cf.* Setchell, *Univ. of Calif. Pub., Botany*, ii, p. 91, 1905 (limu).

[2] H. M. Richards, *Science*, xxi, p. 895, 1905; also H. M. Smith, *loc. cit.*, p. 169.

affinis,[1] together with the non-edible roots of *Inula helenium*, *Dahlia variabilis*, and *Lappa minor* from which inulin itself is isolated.[2] In Japan the most widely used edible products furnished by marine algæ are nori, kanten, kombu, and wakame. A description of these products together with detailed information regarding their manufacture and commercial uses has been published by the Bureau of Fisheries.[3] Their chemical composition has not been the subject of adequate investigation until quite recently. Quantitative determinations of the so-called proximate principles were available, but these give a most imperfect and, as we shall see, often misleading conception of the real chemical make-up and nutritive value of such products.[4] In the commercial marine algæ, starch and the simpler sugars are rarely found. Mannose and mannite may be present; but the materials are primarily characterized by a number of interesting and scarcely investigated polysaccharides which yield dextrose, galactose, mannose, pentose, and methyl pentose on hydration with acids. In addition to this, cellulose also occurs. The fungi and lichens contain similar compounds, chitosan—the polysaccharide yielding glucosamin—occurring in addition as a constituent of the cell wall. To these peculiar carbohydrates the inulin and related compounds obtained from the *Compositæ*, and the xylose-yielding xylan or wood-gum may be added for consideration here.

It is well recognized at present that the only carbohydrates *directly* available in intermediary metabolism are the simple monosaccharides, particularly the six-carbon sugars. If other

[1] *Cf.* v. Noorden. Von Leyden's *Handbuch der Ernährungstherapie*, ii, p. 227.

[2] *Cf.* Dean, *Amer. Chem. Journ.*, xxxii, p. 69, 1904.

[3] *Bull. of the Bureau of Fisheries*, xxiv, p. 133, 1904. References to the literature on these products will also be found in this bulletin. *Cf.* also K. Yendo, *Uses of Marine Algæ in Japan. Postelsia*, 1901.

[4] The most valuable data bearing on the composition of the algæ, lichens, and fungi will be found reviewed by Czapek, *Biochemie der Pflanzen*, i, 1905. On algæ, *cf.* also Koenig and Bettels, *Zeitschr. f. Untersuchung d. Nahrungs- u. Genussmittel*, 1905, p. 487; on lichens, *cf.* Ulander, *Untersuchungen über die Kohlenhydrate der Flechten*, Dissertation, Göttingen, 1905; Ulander and Tollens, *Ber. d. deutsch. chem. Gesellsch.*, xxxix, p. 401, 1906, where reference to the literature, especially to papers by Tollens and his pupils, will be found.

soluble carbohydrates are introduced parenterally into the circulation without previous passage through the alimentary tract, *i. e.* without digestive hydrolysis, most of them are speedily eliminated in large part by the kidneys precisely like some foreign substance. This is true of inulin and the soluble lichen-carbohydrate isolichenin, as well as the more common carbohydrates: glycogen, dextrin, soluble starch or saccharose.[1] From this it appears that the polysaccharides must be subjected to preliminary cleavage into "physiological" sugars before they can become readily available in nutrition. One naturally inquires, therefore, whether and to what extent the animal organism is equipped for appropriate digestion of the complex carbohydrates to which reference has been made above.

The growing evidence of the *specificity* of enzymes, exemplified *par excellence* in such individual carbohydrate enzymes as sucrases, lactases, amylases, etc., at once suggests that mannan, galactan, xylan, pentosan, and all the other complex polysaccharides may require special enzymes for their conversion into assimilable sugars. The experience of Professor Mendel and his collaborators has already brought evidence in this direction. The polysaccharide inulin, apparently so closely related to the readily digested starches, dextrins, and glycogen, and differing only as does "levulan" from "dextran," is not hydrolyzed by the ordinary alimentary enzymes. Neither amylolytically active saliva, pancreatic juice, intestinal extracts nor the vegetable preparation "Taka-diastase" from *Eurotium oryzæ* transform it at all into reducing sugar.[2] It is, however, readily converted into levulose by acid of the strength of the gastric juice at 37° C., even when proteid is present. In accord with the failure of inulin to be digested, Sandmeyer[3] recovered over half of the quantity fed to diabetic dogs in the fæces. Similarly Mendel and Nakaseko[4] failed to get conclusive evidence of glycogen formation in rabbits after inulin feeding, although an accumula-

[1] *Cf.* Mendel and Mitchell, *Amer. Journ. of Physiol.*, xiv, p. 239, 1905.

[2] *Amer. Journ. of Physiol.*, ii, p. xvii, 1898; Richaud, *Compt. rend. de la soc. de biol.*, liii, p. 416, 1900; Bierry and Portier, *ibid*, p. 423; Bierry, *ibid*,, lix, p. 256, 1905; Weinland, *Zeitschr. f. Biol.*, xlvii, p. 286 (footnote), 1905.

[3] Sandmeyer, *Zeitschr. f. Biol.*, xxxi, p. 12, 1894.

[4] Nakaseko, *Amer. Journ. of Physiol.*, iv, p. 246, 1901.

tion is easily procured by using the derived levulose.[1] Inulin is readily converted into soluble sugar by the enzyme inulase (found in fungi such as *Aspergillus* and *Penicilium*) which in turn does not digest starch, although it inverts cane-sugar.[2]

The lichens, formed by the symbiosis of algæ and fungi, furnish a variety of carbohydrates. The most familiar example is the Iceland moss (*Cetraria islandica*), which has already been the subject of investigation in this laboratory.[3] It is composed largely of soluble polysaccharides and yields two characteristic products: lichenin and isolichenin; the latter soluble in water at ordinary temperatures, the former separating in gelatinous form like starch paste. Ulander and Tollens[4] have found that various more common species of lichen differ in respect to the occurrence of these characteristic carbohydrates which are extracted by hot water and gelatinize on cooling. Thus *Cetraria*, *Evernia prunastri*, *Usnea barbata*, afford the similar carbohydrates lichenin, evernin, and usnin; whereas *Cladonia rangiferina*, *Stereocaulon pascale*, *Peltigra aphtosa*, and *Cornicularia aculeata* yield no such polysaccharides. In all the lichens, however, other insoluble polysaccharide residues (mannan, galactan, pentosan, and methyl pentosan) are present in abundance, together with some cellulose. The chemical nature of lichenin has been the subject of controversy; but the evidence that it is a "dextran" rather than a galactan now seems convincing.[5]

It is reported that lichens, especially *Cetraria islandica*, have found wide use as articles of diet.[6] The inhabitants of Iceland, Norway, and Sweden mixed Iceland moss with various cereals and mashed potatoes, from which an "uncommonly palatable and healthful bread was prepared." Sir John Franklin and his

[1] Professor von Noorden lately informed Professor Mendel that he has ceased to recommend the use of inulin-containing foods to diabetic patients. *Cf.* v. Leyden's *Handbuch der Ernährungstherapie*, ii, p. 227.

[2] Dean, *Bot. Gaz.*, xxxv, p. 24, 1903.

[3] Brown, *Amer. Journ. of Physiol.*, i, p. 455, 1898.

[4] *Ber. d. deutsch. chem. Gesellsch.*, xxxix, p. 401, 1906; *cf.* also Müller, *Zeitschr. f. physiol. Chem.*, xlv, p. 278, 1905.

[5] *Cf.* Brown, *loc. cit.*; Ulander and Tollens, *loc. cit.*; Müller, *loc. cit.* The galactan nature of lichenin was advanced by Escombe, *Zeitschr. f. physiol. Chem.*, xxii, p. 295, 1897.

[6] Schneider, *A Text-book of Lichenology*, 1897, p. 23.

companions made use of this lichen during their Arctic voyages. It has especially been recommended as an article of diet for convalescents. Other genera reported to be used as food by Polar navigators are *Umbilicaria* and *Gyrophora*, referred to under the name of Tripe-de-roche or Rock tripe.[1] In view of these facts the digestibility of the specific carbohydrates becomes of considerable interest. Brown[2] found, in this laboratory, that ordinary amylolytic enzymes, as well as hydrochloric acid of 0.3–0.5 per cent. strength have no noticeable effect on lichenin; and isolichenin was at most converted into a dextrin-like form, without production of sugars. These observations have been confirmed in our experiments reported below. In accord with this Brown failed to induce glycogen formation in rabbits by feeding lichenin.

With respect to the digestibility of the more insoluble types of carbohydrates found in the lichens and algæ our knowledge is most limited. Schulze and Castoro[3] have been unable to demonstrate any digestion of hemicelluloses by means of ptyalin, pancreatin, diastase, or "Taka-diastase"; and the existence of enzymes capable of hydrolyzing pentose-polysaccharides in the alimentary tract of man and the higher animals is most uncertain.[4] Pentosans are apparently utilized far better in the alimentary tract of herbivora than in carnivora. To what extent non-enzymatic fermentative processes contribute to this cannot yet be said. Doubtless the cytases and other plant enzymes themselves aid in such utilization.[5] The attempts to obtain evidence of cytases in mammals have for the most part failed. According to Slowtzoff[6] the carbohydrate enzymes of the alimentary tract do not act upon xylan. It is slowly attacked by the acid gastric juice and poorly absorbed. Mannans (salep extract) are likewise not hydrolyzed by animal amylases,[7] and Weinland[8] has been unable to induce the formation of manna-

[1] Smith, *Dictionary of Popular Names of Economic Plants*, 1882, p. 418.
[2] *Loc. cit.*; *cf.* also Berg, *Jahresb. d. Chem.*, 1873, p. 848.
[3] *Zeitschr. f. physiol. Chem.*, xxxvii, p. 50, 1902.
[4] *Cf.* Neuberg, *Ergeb. d. Physiol.*, iii, I, p. 421, 1904.
[5] *Cf.* Bergmann, *Skand. Arch. f. Physiol.*, xviii, p. 119, 1906.
[6] *Zeitschr. f. physiol. Chem.*, xxxiv, p. 181, 1901.
[7] *Cf.* Gatin, *Compt. rend. de la soc. de biol.*, lviii, p. 847, 1905.
[8] *Zeitschr. f. Biol.*, xlvii, p. 280, 1905.

nases in dogs by appropriate feeding. In lower forms, however, cytases and xylanases are reported to occur.[1] In plants these polysaccharide enzymes doubtless arise. Finally the value of unusual sugars such as pentoses, mannose, glucosamin, etc., in nutrition is not yet satisfactorily established; accordingly they deserve little recommendation as actual nutrients.[2] It is interesting, therefore, to find the following comment in Schneider's Lichenology: "In general it may be stated that lichens were used as an article of diet only in case of famine or in those countries where cereals are not abundant, principally because all lichens contain a bitter principle which is very disagreeable to the taste and difficult to remove and which has a deleterious effect upon the digestive tract, producing a form of intestinal inflammation." (p. 23).

EXPERIMENTS.

We have made a study of the digestibility of a number of typical products, viz.:

1. Extracts of *Cetraria islandica* (Iceland moss) containing lichenin.
2. Extracts of *Chondrus crispus* (Irish moss), containing a gelatinizing carbohydrate.
3. Japanese kombu (konbu).[3] Under this name the Japanese recognize several kinds of standard foods prepared from *Laminariaceæ*, especially *L. japonica*. It is ordinarily cooked with soups or served as a vegetable. The carbohydrates which it yields by acid hydrolysis are glucose, fructose, pentose, and methyl pentose.
4. Japanese wakame. From *Undaria pinnatifida*. This alga yields d-galactose in addition to the sugars just mentioned. *Alaria esculenta* is similarly used by the Scotch and Irish.
5. Japanese nori (Asakusanori). Prepared from various species of *Porphyra* and sold in thin, paper-like sheets. It yields especially i-galactose and d-mannose.
6. Agar-agar (kanten). This familiar product is prepared from the

[1] *Cf.* Biedermann and Moritz, *Arch. f. d. ges. Physiol.*, lxxiii, p. 236, 1898; Seillière, *Compt. rend. de la soc. de biol.*, lviii, p. 20, 1905; Pacant, *ibid*, p. 29; v. Fürth, *Vergl. chem. Physiol.*, pp. 190, 226.

[2] *Cf.* Neuberg, *loc. cit.* (pentosans and pentoses); Bial, *Berl. klin. Wochenschr.*, 1905; Ewald, *Festschrift*, (glucosamin).

[3] The modes of preparation of this and the other Japanese seaweed products are described in the *Bureau of Fisheries Bulletin*, No. 562; also by K Yendo, *Postelsia*, 1901. The data regarding the carbohydrates are taken from Koenig and Bettels, *loc. cit.*, p. 457.

hot-water extracts of various species of *Gelidium* and differs somewhat according to its origin. It yields galactose and pentose, the galactan forming about one-third of the dry substance; pentosans about 3.5 per cent.; cellulose, 0.5 per cent.

BEAKER DIGESTIONS. *Methods.* Two preparations of agar-agar were used in the form of a 1 per cent. jelly. The other products were used in finely comminuted form after being boiled with hot water and suspended therein. The enzyme preparations included (a) filtered human saliva, (b) pancreatic juice collected from dogs after injections of secretin, (c) alcoholic (20 per cent.) extracts of dog's pancreas, (d) chloroform-water extracts of dog's and pig's intestine, (e) malt diastase, (f) Taka-diastase (Parke, Davis, & Co.), (g) inulase, prepared from *Aspergillus niger.*[1] The digestive mixtures, preserved with toluene were kept at a constant temperature of 40° C. and tested after twenty hours, and again at the end of three days, for reducing sugars with Fehling's solution. Whenever the reaction was positive a second test for osazones was made with phenylhydrazine. In every case a control trial was carried out under the same conditions with boiled enzyme extracts; the latter experiments were uniformly negative. The trials were often repeated and the numerous protocols need not be recorded here. The results are summarized below:

Ptyalin. The amylolytic power of the saliva used was always previously established with starch paste. The digestion tubes contained the following proportions :

Product used....................	20 c.c. (or 20 gm.)
Saliva..........................	10 c.c.
Toluene.........................	3 drops.

The results of the trials were uniformly negative. Occasionally minute traces of reduced copper precipitate were obtained in the tests after three days' digestion. This was, however, not a constant result. Osazones were never obtained.

Pancreatic Amylase. The tests were made with amylolytically active enzyme solutions. The pancreatic juice was used in proportion of 1.5 or 2 c.c. to 10 c.c. of the carbohydrate material; or 20 c.c. of the latter were mixed with 10 c.c. of the pancreatic extracts. The results were negative in every case.

Intestinal Extract. This contained very active invertin. It was used

[1] For this preparation, active on inulin, I am indebted to Dr. A. L. Dean. For the method of preparation see Dean, *Bot. Gaz.*, xxxv, p. 26, 1903.

in the proportion of 10 c.c. to 20 c.c. of the carbohydrate mixtures. The results were all negative.

Vegetable Amylases. With malt diastase no digestion whatever was obtained in many trials. With Taka-diastase (from *Eurotium oryzae*) the carbohydrate extracts of Iceland moss and Irish moss gave sugar tests after 20 hours' digestion. Usually crystalline osazones could be obtained. The other preparations were not hydrolyzed. Strong solutions of the enzyme were used.

Inulase. The results obtained with this enzyme were comparable with those just reported for vegetable amylases. They suggest interesting questions regarding the specificity of enzymes, especially those of vegetable origin. A specimen protocol is tabulated below:

Digestion Mixture at 40° C. (Toluene, 3 drops.)				Reduction Test with Fehling's Solution.	
				After 20 hrs.	After 3 days
1 % agar,	20 gm.	+2% inulase sol.,	1 c.c.	−	−
Iceland moss,	20 c.c.	"	"	+	+
Irish moss,	20 "	"	"	+	+
Asakusanori,	20 "	"	"	−	trace?
Kombu,	20 "	"	"	−	"
Wakame,	20 "	"	"	−	"
Inulin,	20 "	"	"	+	+

Hydrolysis by Dilute Acid. In order to simulate conditions prevailing in the alimentary tract the extracts were subjected to 0.4 per cent. hydrochloric acid at 40° C. for 20 hours. Traces only of reducing compounds could be produced by this process, quite in contrast with the behavior of inulin, for example, towards acid gastric juice. No definite osazone tests could be obtained. Agar-agar was subjected to the action of dilute acid, then washed to neutral reaction and tested with saliva and diastase. The digestibility was only slightly facilitated by this mild preliminary hydrolytic treatment.

Bacterial Digestion. The action of three cultures of Bacillus coli communis [1] on culture material containing comminuted sea-weed or lichen carbohydrates was investigated. Witte's "peptone" (1 per cent.) was added, the mixtures sterilized, inoculated, and Fehling's test tried at the end of the third and seventh days, with negative results. In one series, Liebig's extract of meat was also added to the extent of 1 per cent. With one of the cultures slight gas production was regularly noted in the media with agar-agar, *Cetraria* and *Chondrus*.

UTILIZATION EXPERIMENTS. *Plan.* The preceding artificial

[1] For these I am indebted to Professor L. F. Rettger.

digestion experiments gave little promise of a successful utilization of the lichen- and alga-carbohydrates in the animal body.[1] Nutrition experiments undertaken to test this point have verified the deduction made. In addition to a standard, easily digested diet, various preparations were fed to dogs or ingested by the author, and the carbohydrate content of the corresponding fæces compared with what the normal control diet alone afforded. The method applied for the quantitative determination consisted in boiling 3 grams of dried fæces with 120 c.c. of 2 per cent. hydrochloric acid for 2 hours. The mixture was then cooled, neutralized, filtered, and made up to 200 c.c. In this fluid reducing sugar was determined by Allihn's gravimetric method, the cuprous oxide being filtered on a Gooch crucible and weighed as cupric oxide. The length of time selected for the acid hydrolysis was decided upon after a preliminary experiment in which various products used for feeding were boiled with 2 per cent. hydrochloric acid and samples removed at intervals for the estimation of sugar The data are given below.

Materials Used (1 gm substance + 100 c c 2 per cent HCl)	Sugar Formed (Estimated as Dextrose) after Boiling		
	1 hour per cent	3 hours per cent	5 hours per cent
Agar-agar (Japanese)	64.1	49.5	46.3
Agar-agar (source unknown)	62.6	54.0	45.3
Agar-agar " "	60.1	47.6	44.2
Agar-agar (recovered from fæces)*	47.3	34.9	32.4
Wakame	8.8	13.0	13.2
Nori	25.5	21.5	23.2
Kombu	20.1	26.9	28.8
Cetraria islandica, dried,	48.2	46.8	44.2
Chondrus crispus, dried,	67.6	74.4	74.1

* The lower figures obtained with this are undoubtedly due to the high ash content of the (impure) preparation.

Feeding Trials. I A small dog was fed on a daily diet of 300 gms of meat On two days 350 c c. and 290 c c respectively of an extract of *Chondrus crispus*, containing 1 per cent. of dry substance and estimated after analysis to yield a total of 4 5 gms sugar, were fed with the meat The fæces of this two-day period were marked off by feeding finely

[1] It is stated in Thorp's *Dictionary of Applied Chemistry* that agar-agar is used to make a paste *not eaten by insects*

ground cork. A comparison of the composition of the air-dry fæces with a sample of both previous and subsequent days was made.

	Sugar by Hydrolysis of Fæces.	Air-dry Fæces per Day.
Fore-period	No reduction test	3 grams.
Chondrus-period	2.7 grams (12.2 %)	11 "
After-period	Trace only	4½ "

No reducing compound was found in the urine. The effect of the *Chondrus* polysaccharides is evident in the increased quantity of fæces as well as the undecomposed carbohydrate present. In this connection it is interesting to note that in unpublished experiments conducted under Professor Mendel's guidance, Mr. Courten failed to induce glycogen formation in rabbits after liberal feeding with *Chondrus* carbohydrates.

II. A small dog received a mixed diet of meat, milk, bread, and cracker meal. On one day 10 gms. of agar-agar were added in the form of jelly. The fæces were marked off as well as possible with lamp-black and analyzed as in Experiment I. The carbohydrate content (as sugar) was as follows:

Fore-period, 2 days............18 per cent.
Agar-period, 1 day.............32 "
After-period...................27 "[1]

III. A small dog was fed with meat. On two days an extract of *Cetraria islandica* containing 2 per cent. of solids was added in quantities of 292 c.c. and 300 c.c. respectively. The carbohydrate content was estimated as equivalent to 6.3 gms. of dextrose. The fæces of the lichen-feeding period were marked off by giving very fine quartz sand at the beginning of the feeding and cork at the start of the after-period. No reducing compound was found in the urine. The composition of the fæces is given below:

Diet.		Composition of the Fæces.		
		Weight, air-dry.	Carbohydrate, as Dextrose.	
		Grams.	Per cent	Grams.
Meat	2 days	10	6.8	0.68
Meat + *Cetraria* extract	2 days	15*	25.8*	3.90*
Meat,	2 days	5*	24.5*	1.20*
Meat,	2 days	6	3.2	0.19

*These fæces belong to the *Cetraria* feeding period.

[1] These fæces doubtless still enclosed some agar-agar, owing to the difficulty of exact separation.

IV. Experiments on a man weighing 53 kilos. This individual (T. S.) is unusually free from intestinal disturbances of any sort. The fæces in all the trials were marked off with lamp-black. On a very liberal mixed diet, including vegetables like corn, the dry fæces contained as much as 7 per cent. of carbohydrate, calculated as dextrose. The results of successive two-day periods on a mixed diet kept approximately comparable follow:

Food.	Fæces.			
	Weight.		Carbohydrate, as Dextrose.	
	Fresh. Grams.	Air-dry. Grams.	Grams.	Per cent.
Usual diet + 20 gm. agar-agar in jelly form	317	51	9.2	18.0
Usual diet, alone	120	29	1.9	6.7
Usual diet + 10 gm. agar-agar	317	43	7.4	17.2

Since the agar-agar used yielded over 50 per cent. of reducing carbohydrates it will be noted that the utilization of the polysaccharides was very imperfect. The effect on the total mass of fæces passed is also very marked. The agar easily retains water in the alimentary residues and prevents the formation of dry, hard, fæcal masses which readily induce constipation. This property of the agar, together with its failure to dissolve readily by digestion or fermentative change has led Professor Mendel to suggest its use in appropriate cases of chronic constipation, with very satisfactory results. The same suggestion has lately been advanced by Schmidt,[1] without experimental evidence of the kind here presented.

The results of another series of observations extending over eighteen days are summarized below. The individual periods each covered two days, the fæces being marked off correspondingly with lamp-black. The daily diet, selected in accord with the subject's preference, consisted uniformly of

[1] *Münch. med. Wochenschr.*, 1905, p. 1970.

Beef steak, 513 grams.
Bread, 500–600 grams.
Sugar, 40 grams.
Butter, 31 grams.
Three eggs.
Two apples.

In alternate periods the algæ preparations were added in boiled form, the quantity being distributed through the three meals. Nitrogen estimations were made by the Kjeldahl-Gunning method.

UTILIZATION EXPERIMENTS ON MAN.

Substance added to the Diet.	Urine			Fæces					
				Weight		Nitrogen		Carbohydrate (as Dextrose)	
	Volume c.c.	Specific Gravity	Nitrogen Grams	Moist Grams	Air-dry Grams.	Air-dry Grams	Air-dry Per cent	Air-dry Grams.	Air dry Per cent
Agar-agar, 19 gm " " 5 "	1275 1450	1 027 1 026	20 3 24 8	313	62	4 1	6 6	8 8	14 4
None None	1315 1930	1 026 1 017	24 3 25 7	222	46	3 6	7 7	0 7	1 6
Wakame, 20 gm " 20 "	1570 1600	1 025 1 026	24 8 26 6	321	70	4.9	7 0	3 4	4 8
None None	1400 1440	1 028 1 026	23 5 25 3	275	55	4 4	8 0	0 7	1.4
Asakusanori, 6 gm " 6 "	1530 1615	1 024 1 025	23 7 23 1	207	52	4 3	8 3	trace	0 1
None None	1400 1255	1 024 1 025	23 4 22 8	171	43	3 4	8 0	trace	0 1
Kombu, 25 gm. " 20 "	1650 1610	1 024 1 024	25 4 25 7	234	59	4.7	8 0	2 5	4 2
None None	1810 1460	1 023 1 025	23 4 24 8	168	41	3 9	9 6	trace	0 1
Raw "Italian" Chestnuts (*Castanea*) 150 gm. " " " 68 "	1495 1420	1 024 1 025	24 8 24.7	233 76	29 21	2 0 1 8	7 0 8 5	4.9 0 7	16 9 3 2

The experiment with unboiled chestnuts was added to ascertain the effect of raw starch on the composition of the fæces in comparison with algæ carbohydrates. The results indicate the relative indigestibility of starch offered in this form.

Character of the Unabsorbed Carbohydrates in the Fæces. In the experiments with agar-agar an attempt was made to recover

the unused material where a considerable quantity of fæces was available. They were extracted with hot water and the extracts precipitated with alcohol. The precipitate was redissolved in hot water and allowed to gelatinize on cooling. The pieces of solid jelly were soaked in water to remove soluble salts and other carbohydrates, then redissolved in hot water and again precipitated with alcohol. The products were characteristic in respect to solubility and gelatinizing power; but as the analysis (p. 259) shows, they were still relatively impure. Normal fæces failed to give any agar-like carbohydrate.

CONCLUSIONS. A survey of the protocols indicates the poor utilization of the Japanese preparations—a result which our preliminary studies on digestibility lead one to anticipate. The polysaccharide carbohydrates of such products as we have examined are scarcely attacked by familiar alimentary enzymes or profoundly altered by such bacterial processes as they might be expected to become subject to in the digestive tract of man. It is an interesting observation that some of the enzymes of plant origin seem to be somewhat more effective in liberating sugars from these polysaccharides. The exhaustive review of Japanese nutrition investigations recently published by Professor Oshima[1] contains references to several studies on the utilization of the nutrients of dietaries rich in algæ food preparations. The compilation of one hundred and twenty-five nutrition investigations on Japanese shows the following average coefficients of digestibility (p. 54):

Kind of Food.	Utilization of		
	Protein. Per cent.	Fat. Per cent.	Carbohydrate. Per cent.
Ordinary mixed diet	87	85	98
Vegetable mixed diet	78	82	98

In contrast with these figures are the results obtained in trials with algæ as food (p. 194.):

[1] *U. S. Dept. of Agric., Office of Exper. Stations, Bull. No. 159, 1905.*

Dried Marine Algæ.	Coefficient of Digestibility.	
	Carbohydrates, Including Crude Fibre.	Crude Fibre.
Ecklonia bicyclis	36.2	17.8
Laminaria sp.	75.2	55.3
"	55.0	65.4
Ulopteryx pinnatifidæ	72.8	2.8

Such experiences warn us against the extravagant claims for some of the modern food products manufactured from marine algæ.[1] As food accessories they doubtless have a deserved place and they may well serve uses quite apart from any nutrient functions. But at the present time they can scarcely be rated as effective foodstuffs. It has been claimed that some of the species rich in nitrogen, *e. g.*, *Porphyra*, are particularly valuable because of their content of proteid. The latter is, however, merely calculated on the basis of the nitrogen content. The experience gained in the case of the mushrooms where, according to Mendel,[2] the nitrogen is largely in the form of unavailable non-proteid compounds, has indicated what erroneous inferences may be drawn regarding the nutritive values of plant substances rich in nitrogen. Until more is known regarding the character of the nitrogenous constituents of the algæ, it seems best to reserve judgment on this point. The number of algæ susceptible of being prepared in palatable ways is doubtless very large; perhaps it is after all not without significance that in this country they have not found favor in competition with an abundance of wholesome and more distinctly nutritive food materials.

SUMMARY.

Experiments with a variety of alga and lichen preparations containing a large proportion of polysaccharide carbohydrates

[1] Some of these preparations are sold as substitutes for animal gelatin. One widely used agar product which we have examined contains the statement on the package: "Its food value is more than double that of an equal weight of eggs or beef-steak."

[2] *Amer. Journ. of Physiol.*, i, p. 225, 1898.

indicated that the latter were not readily transformed to sugar by carbohydrate-digesting enzymes of animal origin and scarcely more readily by vegetable enzymes or bacteria. Corresponding with this, the digestibility and availability of such products in the alimentary tract were found to be very imperfect in both man and animals. The results of these investigations should be applied in criticism of the claims made for some of the "food preparations" rich in indigestible carbohydrates, and many food materials more properly rated as "food accessories." Incidentally the basis for certain therapeutic uses of sea-weed preparations has been indicated

I desire to acknowledge the help and criticism of Professor Mendel, who suggested this study to me.

ON THE SEPARATION OF INDOL FROM SKATOL AND THEIR QUANTITATIVE DETERMINATION.

By C. A. HERTER and M. LOUISE FOSTER.

(Received for publication, July 10, 1906.)

In a recent publication[1] it was pointed out that the presence of indol in a solution can be detected by means of β-napthaquinone-sodium-monosulphonate, and that owing to certain properties possessed by the compound formed by the union of indol with this napthaquinone compound it is possible to determine with considerable accuracy the quantity of indol in solution. As indol is sometimes associated in the course of putrefaction with skatol (this is not infrequently the case with the contents of the large intestine) it becomes desirable to have a method for the separation of indol from skatol. These putrefactive decomposition products may be separated by means of their picrates, but the method involves so much time as to make it unfit either for clinical or ordinary chemical investigations. It is believed that the method about to be described will prove practically helpful in effecting the rapid and nearly complete separation of indol from skatol and that it will further serve for the determination of the quantity of skatol present.

The method is based on the fact that by means of the napthaquinone compound above mentioned, it is possible to remove the indol almost completely from a solution containing both indol and skatol, and that the skatol remaining after the removal of the indol can be distilled and recognized by means of the dimethylamidobenzaldehyde reaction described by Ehrlich.

If one takes a putrefactive mixture containing both indol and skatol, these bases should first be distilled either in acid or alkaline solution, sometimes preferably with the aid of steam. In the distillate the skatol passes over earlier than the indol, as can be easily shown by means of the blue color which it gives on boiling with Ehrlich's aldehyde. To the distillate containing

[1] "A Method for the Quantitative Determination of Indol." This Journal, i, p. 257, 1906.

the indol enough sodium or potassium hydroxide is added to render it slightly alkaline. An excess of the β-napthaquinone-*sodium-monosulphonate is now added to this solution.* This substance in the course of a few minutes reacts almost completely with the indol present but not with the skatol, and the result of the reaction is the appearance of the blue or purplish-blue precipitate of the newly formed indol naphthaquinone compound, which may be removed by filtration. In cases where the concentration of indol is too small to give rise to a precipitate when treated with the napthaquinone compound the solution simply develops a green or greenish-blue color. The solution is now acidified and subjected to distillation (with or without the use of steam). The skatol passes over into the distillate; the indol is held back in the form of the napthaquinone compound with the exception of a very small uncombined portion which passes over with the skatol. The amount of indol, however, which passes over after treatment with naphthaquinone is so small that it is practically negligible, although its presence is detectable through the red color which it gives when acted upon by dimethylamidobenzaldehyde. The distillate containing skatol is boiled with a solution of Ehrlich's aldehyde in sulphuric acid.[1] A slight amount of dilute hydrochloric acid is now added and has the effect of intensifying the blue color produced by boiling the skatol with the Ehrlich aldehyde solution. A little experience is required to find the amount of hydrochloric acid which gives the maximal intensification of the reaction. An excess of hydrochloric acid causes the blue color to fade. It is important to use an excess of the Ehrlich aldehyde solution in order to develop fully the color reaction with skatol. The color obtained through the action of Ehrlich's aldehyde upon skatol is purple-blue rather than blue so long as the solution is hot. On cooling it under the tap the blue color asserts itself more strongly and the solution may become somewhat opalescent from the separation of uncombined dimethylamidobenzaldehyde. Chloroform is now added to the solution containing the blue product. On agitation with the solution this carries out the blue color, and the chloroform assumes a pure blue tint. By means of a good colorimeter the quantity

[1] Five per cent. aldehyde in ten per cent. sulphuric acid.

of skatol present in the original solution may be approximated by the intensity of the color reaction.

On evaporating the chloroform containing the blue color resulting from the action of dimethylamidobenzaldehyde on skatol one obtains an amorphous blue material which can be partially purified from the admixture with Ehrlich's aldehyde by the use of petroleum ether. The nature of this compound is not at present known. The melting-point of our preparation lay between 65° C. and 66° C.

Apparently few micro-organisms growing on bouillon are capable of making skatol, at least within a week or ten days, although many bacteria are able in the same length of time to produce considerable indol. It was found that by inoculating an asparagus-bouillon medium with mixed flora from the feces of a normal pig or from certain human fecal material considerable skatol was formed in the course of two weeks.[1] From a putrefactive mixture obtained by the action of pig's feces, 17.29 milligrams[2] of skatol were recovered from 244 cubic centimeters of the asparagus-bouillon medium—a very large yield of skatol. The separation of skatol from the culture was in this case easily effected by means of the method outlined, and the blue compound obtained in chloroform solution was indistinguishable from that obtained from a pure solution of skatol.

In another instance in which indol was present in a culture containing a larger quantity of skatol than indol, an equally satisfactory separation was made.

The method of separating indol and skatol here described has been used in a routine way during the past eight months in connection with the study of the feces and has given satisfaction. To twenty-five grams of the material has been added twenty cubic centimeters of water and one to two cubic centimeters of a ten per cent. sodium hydroxide solution. The suspension is then subjected to distillation with the aid of steam until the distillate no longer gives a color reaction when boiled with the

[1] The exact conditions that must prevail to secure this large formation of skatol have not been determined and difficulty has been met with in duplicating these results at will.

[2] This result was reached by a colorimetric observation. Other observations on the same material gave the following fairly concordant results: 17.87 mg., 18.62 mg., 18.62 mg., 19.29 mg.

dimethylamidobenzaldehyde solution. Ordinarily the indol and skatol present go over completely within an hour, but where the material develops frothing a more prolonged distillation may be necessary. When the distillate has been obtained, it is treated in the mannner above outlined for the separation of indol and skatol. In some cases one obtains only skatol from the feces, but as a rule indol is also present. In the presence of indol the chloroform extract, instead of being a pure blue, may have a slightly purplish tinge, owing to unavoidable admixture with a slight amount of indol.

It is important in making the colorimetric estimations of the quantity of skatol present to employ a standard color solution for comparison with the color obtained from the distillate containing the skatol. Various dyes have been tried with a view to obtaining a standard solution which will retain its color unchanged. Experience has shown that the best standard color solution is one obtained from a solution of skatol. Although such a solution fades after a few days, especially when exposed to the light, and may assume a greenish tint, in the dark it may last several weeks without undergoing appreciable change. Moreover as the skatol standard solution is readily prepared, there is little disadvantage from being compelled to renew the color solution from time to time. This solution may be conveniently prepared by dissolving five milligrams of skatol in water and acting upon it with an excess of dimethylamidobenzaldehyde. It commonly requires from one hundred to one hundred and fifty cubic centimeters of chloroform to extract completely the blue coloring matter which has already been described. The quantity of coloring matter present is sufficient to impart a deep blue color to this volume of chloroform. Ten cubic centimeters of this solution are placed in the receptacle of the Duboscq colorimeter and used as a standard for comparison with the chloroform color solution obtained from the distillate to be tested. The matching of the colors can usually be made very closely. In cases where the quantity of skatol is so small that the trace of indol present influences the color of the chloroform solution, changing it to violet or even purple, it is more difficult to obtain a satisfactory matching of colors. In this case it may be necessary to add a small quantity of indol to

the skatol employed in making the standard color solution. This then imparts to the standard color solution a violet tint like that obtained from the distillate to be matched. It seems unnecessary to give further details. After some experience with the method of matching colors it is possible to employ the method so that it will give satisfactory quantitative results.

ON THE POISONS OF AMANITA PHALLOIDES.

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(Received for publication, August 6, 1906.)

It has recently been shown by one of us (W. W. Ford[1]) that the poisons of the fungus *Amanita phalloides*, the variety responsible for the great majority of deaths from mushroom intoxication, belong to the group of bacterial toxins. They were thus classified in virtue of their causing characteristic lesions in animals after a definite latent period, and because an *immunity* may be established toward them in susceptible animals after the administration of non-lethal doses. The serum obtained by Ford from his immunized animals was antihæmolytic and antitoxic in character; it prevented the solution of blood corpuscles *in vitro* and neutralized the poisonous action of the fungus on animals.

Ford[2] also proved that the hæmolytic principle first described by Kobert under the name phallin, as the only poison present in the fungus, is in fact accompanied by a highly toxic, thermostabile substance devoid of hæmolytic properties, whose presence can be demonstrated by biological experiments. For this thermostabile substance an antitoxin[3] can be produced devoid of any antihæmolytic action and Ford has proposed for it the provisional name Amanita Toxin.

In view of the importance that attaches to the physical and chemical properties of toxins in elucidating questions of immunity it was determined to make a chemical study of the poisons of *Amanita phalloides*.[4]

[1] *Journ. of Infect. Dis.*, iii, pp. 192–224, 1906.

[2] *Journ. of Exper. Med.*, viii, pp. 437–450, 1906.

[3] *Loc. cit.*, p. 219.

[4] It was only after this paper was partly written that we learned of Kobert's announcement in 1899 that he had discovered a second poison with alkaloidal properties in the fungus in question. That this paper

PREVIOUS CHEMICAL ANALYSES.

The earliest investigation of a chemical nature on the active principles of poisonous fungi is probably that of Letellier[1] who in 1826 obtained from a considerable variety of fungi (*A. muscaria*, *A. phalloides*, and *A. verna*) a highly resistant body which he called Amanitin. Subsequently Letellier and Speneux[2] obtained two poisons from a fungus found in the vicinity of Paris and known as *Hypophyllum crux militense* (Paulet), probably a variety of *Amanita*. One of these poisons, which was obtained from both alcoholic and aqueous extracts, caused in cats a violent inflammation of the mucous membrane of the alimentary canal, with vomiting and diarrhœa; the other poison was very resistant, withstanding boiling and treatment with acids; it also had the capacity after boiling with weak sulphuric acid of reducing copper solutions. This latter substance is characterized as a *glucosidal alkaloid*, identical with the Amanitin originally described by Letellier. The action of this substance is described as being purely narcotic.

Boudier[3] in 1866 attempted to give a methodical analysis of the constituents of *Amanita phalloides*. As present in the fresh juice he names glucose, albumin, a mucilaginous substance, viscosine, a hygroscopic viscous substance, mycétide, tannin, citric acid, malic acid, and their salts, fats and oils and inorganic salts, as potassium chloride and calcium phosphate. None of these substances are described as being toxic, and, indeed, his methods of treatment were such that his products could not have retained their toxicity. At one stage of his operations he ob-

eluded our earlier search is accounted for by the fact that it appeared in a Journal found in but few libraries and that it is not referred to in such books as Kunkel's *Handbuch der Toxikologie* although this author was engaged in a controversy with Kobert. Kobert's statements in regard to this alkaloid appear in an address published in the *Sitzungsberichte der naturforschenden Gesellschaft zu Rostock*, p. 26, 1899, Anhang to the *Archiv des Vereins der Freunde der Naturgeschichte in Mecklenburg*, liii, 1899, II Abtheilung. We have now obtained the original paper and reference will be made to it later.

1 Thèse de Paris, 1826.

2 *Annales d'hyg. pub. et de méd. leg.*, p. 71, 1867.

3 *Des champignons au point de vue de leurs caractères usuels, chimiques et toxicologiques*, 1866.

tained a small quantity of an impure residue which was supposed to contain the neutral salt of an alkaloid. He reports that experiments with this material on mice were inconclusive in their results. Boudier named this assumed alkaloid *bulbosine*, but he was unable on account of lack of material to isolate it or even to establish its presence to the satisfaction of subsequent workers.

Oré[1] in 1877 experimented with extracts made from both fresh and dried fungi and concluded on biological grounds alone that the toxic principle of the fungus is an alkaloid "qui offre avec la strychnine *une parenté physiologique incontestable.*" To this hypothetical substance Oré gave the name *phalloidin.*

From this time on, little of importance was accomplished until Kobert[2] in 1891 made the important observation that aqueous and saline extracts of dried *Amanita phalloides* contain a substance which rapidly dissolves the blood corpuscles of a variety of animals even in dilution of 1-125,000 of the dried material. This substance, which Kobert named phallin, he regarded in his earlier communication as the sole toxic constituent of the fungus in question. As contained in simple extracts, Kobert found phallin to be a highly unstable substance, easily rendered inactive by contact with acids, alcohol, and other agents, and by exposure to a temperature of 70° C. His extracts contained a little coagulable proteid and the similarity of this unstable hæmolytic substance to a hæmolytic poison secreted by certain spiders (*Spinnengift*), which Kobert also believed to be proteid in character, led him to assert that the blood-laking poison of *Amanita phalloides* is a *toxalbumin.*

It must be clearly pointed out that phallin was not *isolated* by Kobert and that his statements in regard to its chemical nature rest on inference alone. Our own observations lead us to believe that Kobert's characterization of this hæmolytic substance as a toxalbumin is entirely erroneous.

A few years later Seibert,[3] working in Kunkel's laboratory with *Amanita citrina*, a variety of *A. phalloides*, was unable to demonstrate any hæmolytic substance in this fungus although it was a definitely toxic to certain animals.

[1] *Arch. de physiol. norm. et path.* (II), xl, p. 274, 1877.
[2] *St. Petersburger med. Wochenschr.*, xvi, pp. 463, 471, 1891.
[3] Inaug. Dissert., München, 1893.

Kunkel[1] concludes that the phallin of Kobert, while very interesting from the pharmacological point of view, plays no rôle in recorded cases of poisoning, since on the basis of his pupil Seibert's work he believes that this principle is not present in the juice of the fresh fungus (*A. citrina*), and furthermore, even if present, it would be rendered inert by a temperature far below that at which mushrooms are cooked. Finally, neither clinical symptoms nor the pathological lesions in recorded cases of poisoning can be brought into agreement with the action of any known hæmolytic substance.

Bourquelot,[2] while accepting phallin as being the hæmolytic and poisonous principle of the fungus, is not convinced that it is a toxalbumin since the fungi remain poisonous after cooking while toxalbumins lose their activity at 70° C.

Finally, in 1899, Kobert[3] in his second communication upon this subject noted that after precipitating aqueous extracts of the fungus with alcohol the clear filtrate contained a highly active poison without hæmolytic properties. This he stated to be an alkaloid.

This poison also was not isolated by Kobert. He limits himself to the statements that it is insoluble in ether, soluble in alcohol, and "gives precipitates with certain alkaloidal reagents." As these stetements apply equally well to many plant extracts which contain non-nitrogenous glucosides, citrates, chlorides, pigmentary and resinous substances, but no alkaloid, it cannot be claimed that they throw much light on the chemical nature of this second poison.

OUR METHOD OF PREPARING A HÆMOLYTIC EXTRACT.

About 50 grams of dried *A. phalloides*,[4] which had been accurately identified as such in the fresh state, were crushed in a mortar and then mixed with 300 c.c. of distilled water. After

[1] *Handbuch der Toxikologie*, ii, p. 1048, 1901.

[2] Richet, *Dict. de physiol.*, iii, 1898, Art., Champignons.

[3] Journal cited in footnote to p. 273.

[4] In all cases the fungi employed were identified by the help of standard publications on mycology, especially those of Atkinson, Farlow, and Peck. Only the pure white *A. phalloides* or *A. verna* was used, the yellow variety, frequently called *A. citrina*, and specimens with a brownish or grayish pileus being reserved for special investigation.

standing on ice for two days the mixture was strained through linen and the fluid thus obtained passed through filter paper and then through a Berkefeld candle. This process was repeated in order to remove completely the poisons from the dried material.

The combined filtrates from the Berkefeld candles, usually amounting to between 600 and 700 c.c., were concentrated under reduced pressure at a temperature not exceeding 35° C., and to the concentrated extract absolute alcohol was added in small quantities at a time till no further precipitate was found. This precipitation occurs rapidly and the supernatant alcohol should be poured off quickly to avoid any but the briefest contact. The precipitate thus formed contains the hæmolytic substance while the straw-colored alcoholic filtrate contains the deadly Amanita toxin. This filtrate was concentrated immediately under reduced pressure until freed from alcohol.

FURTHER TREATMENT OF THE IMPURE HÆMOLYSIN.

The precipitate containing the hæmolysin can be further purified by solution in a small quantity of water and by precipitation with "Columbian spirits."[1] This precipitation must be effected as quickly as possible and the alcohol then removed, as experience has shown that prolonged contact with methyl alcohol is decidedly injurious to the hæmolytic activity of our substance. The flocculent or granular precipitate is dried *in vacuo* over sulphuric acid. When dry it consists of brown scales and lumps so hygroscopic in character that exposure to air soon causes them to assume a tarry consistency. This material is powerfully hæmolytic. It contains, so far as our observations show, a very small amount of proteid, a pigmentary substance, inorganic salts, and a *glucoside* which constitutes by far the greater bulk of the precipitate.

The presence of proteid can best be demonstrated as follows. A considerable quantity of the precipitate just described is dissolved in a small quantity of water, thymol is added, and the solution is dialysed in a collodion sac for forty-eight hours. The sac is renewed three or four times

[1] It seemed to us that this commercial preparation of methyl alcohol which contains a little free acid of organic nature is more effective as a precipitant than pure methyl alcohol of the same strength (93 to 97 per cent.).

during the progress of the dialysis. The pigmentary substances which obscure proteid reactions are thus largely removed. Trichloracetic acid in substance is now added to the contents of the sac till no further precipitation occurs. The turbid mixture is transferred to a separatory funnel and shaken with an equal volume of ether. As a result the precipitated proteid collects at the boundary between the ether and the water and may easily be obtained upon a filter. It is then found to be so far free from pigment that it responds in the most satisfactory manner to the biuret reaction and to Millon's test. The color obtained in this biuret reaction was the fine violet characteristic of native proteids. The dialysates were found to be free from proteids both of the coagulable and non-coagulable class.

ON THE REMOVAL OF PROTEID FROM THE METHYL ALCOHOL PRECIPITATE WITHOUT LOSS OF HÆMOLYTIC ACTIVITY.

Having thus shown that proteid is present in the methyl alcohol precipitate, it remains to determine whether it can be removed from this precipitate without materially lessening its hæmolytic activity. We have found that this may be accomplished by at least two methods, involving the use of metaphosphoric acid and of uranyl acetate.

METHOD I. METAPHOSPHORIC ACID. *This acid must be freshly prepared and used at once* to ensure satisfactory results. Phosphorus pentoxide is spread out in a thin layer on the bottom of a wide-mouthed Erlenmeyer flask, and a plug of moistened filter paper is then suspended in the mouth of the flask. In half an hour or less the metaphosphoric acid is ready for use. By means of a glass rod a little of the thick viscous acid is added to an aqueous solution of the methyl alcohol precipitate until the proteid is completely coagulated. The proteid material is removed[1] by means of a small filter charged with absorbent cotton, and the filtrate is again tested with metaphosphoric acid. After this treatment our solution gives no turbidity when poured upon crystals of trichloracetic acid.

This proteid-free solution after neutralization with sodium bicarbonate to cochineal as an indicator is found to have retained its hæmolytic activity practically unaltered. Control experiments with similar quantities of metaphosphoric acid, also

[1]Neither here nor when uranyl acetate was used did we wash the precipitate.

neutralized with sodium bicarbonate, had absolutely no injurious effect on red corpuscles.

In proof of the above statement that our hæmolytic solution is now entirely free from proteid material capable of giving the biuret reaction we may cite the following experiment.

After treatment with metaphosphoric acid as above described, the neutralized solution was treated with lead acetate, until precipitation was almost complete, was filtered, and the filtrate completely precipitated with solution of basic lead acetate. The two precipitates were then separately suspended in water and each decomposed with hydrogen sulphide. After filtering off the lead sulphide, the filtrates were separately concentrated to a small volume. With neither solution could the biuret reaction be obtained. We thus see that freshly prepared metaphosphoric acid is a complete precipitant for native proteids.

Method II. Uranyl Acetate. The methyl alcohol precipitate containing the hæmolysin was dissolved in water made faintly alkaline with sodium carbonate and then treated with a saturated solution of uranyl acetate until no further precipitate was obtainable. During the precipitation care must be taken that this mixture remains nearly neutral in its reaction toward litmus, as more than a trace of free acid is decidedly injurious to the hæmolysin.

After removing the precipitated proteid by filtration, the excess of uranyl was removed from the filtrate by treating it with a solution of disodium hydrogen phosphate. The filtrate from the uranyl phosphate showed no diminution in hæmolytic activity as compared with the original solution. Our second method leads us to the same conclusion that the small amount of proteid originally present in our methyl alcohol precipitate has nothing to do with its hæmolytic activity.

It may be stated here that Kowalewsky[1] has shown that uranyl acetate will completely remove from various albuminous fluids every trace of proteid giving a biuret reaction, and that Jacoby[2] and others have used this reagent for the removal of proteids from faintly alkaline solutions.

We have thus been able to show that proteid may be precipi-

[1] *Zeitschr. f. anal. Chem.*, xxiv, p. 551, 1885.
[2] *Zeitschr. f. physiol. Chem.*, xxx, p. 135, 1900.

tated from our hæmolytic solutions without appreciable loss of their power to dissolve red blood corpuscles. We feel justified in asserting, therefore, that this hæmolytic principle is not a *toxalbumin* and that Kobert was in error when he characterized it as such.

We do not forget that there are substances found among the end products of digestion (peptoids, peptids) and in the urine (oxyproteic and uroproteic acid) which no longer give the biuret reaction, but which on chemical grounds are to be regarded as closely allied to the true proteids. Substances of the first class which might possibly be formed by autolysis in drying fungi or even in the regressive metabolism of these plants may be eliminated on the ground that they are more resistant in their behavior toward chemical reagents (acids) and heat, more soluble in weak alcohol, and diffuse more rapidly through a collodion sac than does our hæmolysin. Substances of the second class (uroproteic acid, oxyproteic acid) are precipitated by cupric acetate, which reagent fails to precipitate the hæmolysin. Furthermore, both Kobert and Ford have shown that the hæmolysin loses its activity on exposure to those digestive ferments which would have no further action on substances of the peptoid class.

Criticisms of the above nature, however, lose their force in view of the facts presently to be adduced, which show that the hæmolytic substance is a glucoside. This new point of view, again, is confirmed by the results of the experiments with ferments. We have found that the hydrochloric acid alone of a pepsin-hydrochloric mixture will deprive the hæmolysin of its blood-laking power, so that it becomes unnecessary now to attribute any action to pepsin itself. In reference to the action of pancreatin, it must be borne in mind that this product constitutes a mixture of ferments which is known, for example, to be capable of liberating glucose[1] from triacetyl glucose, and it is not surprising that it should also effect the decomposition of our unstable glucoside. Kölliker and Müller[2] have found that pancreatic juice has the power of decomposing the glucoside amygdalin, and Fischer and his pupils have shown that it can effect the hydrolysis of various synthetically prepared esters. It will later be shown that pancreatic juice also deprives our hæmolytic glucoside of its blood-laking power.

[1] Acree and Hinkins, *Amer. Chem. Jour.*, xxviii, p. 370.
[2] Cited from Green, *The Soluble Ferments and Fermentation*, 1899, p. 147.

HÆMOLYTIC PROPERTIES OF FRESH FUNGI.

It has already been stated that Seibert, working in Kunkel's laboratory, was unable to demonstrate any hæmolytic activity in poisonous extracts of freshly gathered *A. citrina* and that Kunkel therefore concluded that the hæmolytic substance described by Kobert is not the active agent to which recorded cases of Amanita poisoning can be referred.

In view of this difference of opinion among authors we have recently made some observations on perfectly fresh specimens of *A. phalloides* (white variety). Aqueous extracts were made within a few hours after gathering the fungi. Three small plants were crushed in a mortar with 15 c.c. of distilled water. The perfectly colorless but slightly turbid filtrate was powerfully hæmolytic, one drop of the fluid when diluted one hundred times sufficing to lake completely within an hour 1 c.c. of a 5 per cent. suspension of rabbit's blood. Hæmolysis occurs very rapidly with large quantities of the undiluted juice, but here secondary changes occur due to the acidity of the extract. If, however, the extract be first neutralized, the laking of the blood occurs in as perfect a manner as with the diluted but non-neutralized extract. Similar observations were made with the fungi from various localities and we feel justified, therefore, in asserting that the Amanita-hæmolysin is present in large amount in the freshly gathered fungi.

From this extract of the fresh fungi the proteids were also removed by precipitation with freshly prepared metaphosphoric acid and with uranyl acetate, the necessary precautions in the use of these reagents being duly observed. The resulting proteid-free solutions were perfectly colorless, as clear as water, and powerfully hæmolytic, showing no noticeable loss of activity.

To some of the undiluted fresh extract hydrochloric acid to the extent of three tenths of one per cent. was added and the mixture allowed to stand in the thermostat for two hours at 37.5° C. At the end of this time the mixture was practically devoid of hæmolytic activity. A second experiment in which pepsin was used in addition to the hydrochloric acid led to the same result. These experiments show how extremely susceptible the hæmolysin is to the action of free acids even in great dilution.

The hæmolysin is also injured by contact with weak alkalies but more slowly than with acids. To some of the undiluted fresh extract sodium carbonate was added until it had attained the strength of an $\frac{N}{8}$ Na_2CO_3 solution. Protected with thymol, the extract was then placed in a thermostat (37.5° C.) together with a control tube containing only extract and thymol. After remaining for seventy-two hours in the thermostat, the extract containing the sodium carbonate showed a decided diminution in hæmolytic power. Three drops added to 5 c.c. of a 5 per cent. suspension of rabbit's blood showed no indication of hæmolysis until ten minutes had elapsed, while the same quantity of the native extract from the control tube effected the laking of blood almost instantly.

To 2 c.c. of the fresh extract containing thymol and the above-named amount of sodium carbonate 0.5 c.c. of pancreatic juice obtained from the dog after the injection of secretin was added and the mixture placed in the thermostat. After twenty-four hours the hæmolytic power of the extract was greatly diminished and after forty hours it was found to have entirely lost its blood-laking power. We have shown that the hæmolysin is not an albuminous substance and we see in this experiment a confirmation of our opinion that it is, in fact, a glucoside and one which is capable of being decomposed by one of the ferments of pancreatic juice. We hope later to throw some light on the true nature of this ferment action.

While the foregoing experiments with fresh fungi were all made with *A. phalloides*, we may add that we have lately found that the juice of fresh specimens of *A. citrina*, as growing in the vicinity of Baltimore, is also powerfully hæmolytic.

It seems hardly possible that Seibert could have failed to demonstrate this property in his fresh extracts of *A. citrina* had it been present, and we can only conclude, in view of his negative results, that specimens of this fungus growing in different parts of the world may differ widely as to hæmolytic activity.

We have thus shown that the Amanita-hæmolysin is present in fresh specimens both of the white and of the yellow variety of the fungus and we are of the opinion that, in consequence of its great susceptibility to acids, it is under all ordinary conditions destroyed in the stomach if it had not already become

innocuous through cooking and plays no rôle whatsoever in mushroom poisoning. This opinion finds support in the absence of hæmoglobinuria and of pigmentation of the spleen, two of the characteristic signs of intoxication from hæmolytic agents.

The Amanita-toxin, on the other hand, is far less susceptible to the action of weak acids and heat. Oré found that weak solutions of acetic acid will extract a deadly poison from both dried and fresh specimens of Amanita, so that the residue becomes edible and harmless. The acetic extract, however, contains the poison with its virulence apparently unaltered. Ford has shown that this toxin will withstand prolonged heating and digestion with pepsin-hydrochloric acid and pancreatin. We are therefore forced to conclude that our second poison, the Amanita-toxin,[1] is from a practical point of view, *the* poisonous principle of the fungus, in a word, the only poison which is operative in man after the ingestion of this deadly plant.

NATURE OF THE HÆMOLYTIC SUBSTANCE.

The chemical instability of this substance renders its isolation and purification a matter of great difficulty, and we do not claim that we have yet obtained a product pure enough for elementary analysis. Nevertheless, we believe that the following experiments throw much light upon the nature of the hæmolysin and indicate that its identification is now possible.

Treatment with Basic Lead Acetate. Although Kobert states that the hæmolysin will not endure treatment with lead acetate and subsequent removal of lead with hydrogen sulphide, nevertheless we have met with partial success in applying this method of isolation. The methyl alcohol precipitate from 25 grams of dried fungi was precipitated with basic lead acetate, the precipitate was thoroughly washed with a dilute solution of the acetate and then suspended in water and treated at room temperature with a solution of sodium sulphate. After the precipitate had been repeatedly shaken with this solution it was found that little, if any, decomposition had taken place. The precipitate was

[1] We wish again to emphasize the point that in thus speaking of the Amanita-toxin as a single poison we are guided by the biological experiments of Ford; we are well aware that further chemical investigations may show that this toxin consists of more than one poison.

then freed from sodium sulphate, suspended in much cold water, and decomposed with hydrogen sulphide. After removal of the lead sulphide, the excess of hydrogen sulphide was driven off by means of a current of hydrogen and the fluid neutralized with sodium carbonate to cochineal as an indicator. The solution was then concentrated to a small volume at a temperature not exceeding 35° C. The concentrated solution was definitely hæmolytic, although much reduced in activity. From this solution the hæmolysin was precipitated with ethyl alcohol, immediately freed from alcohol, and dried *in vacuo* over sulphuric acid. When dry it consisted of hard, gray lumps which rapidly absorbed moisture on exposure to air and which in smallest quantity gave all the glucosidal reactions to be later described. This material also exhibited hæmolytic activity, though not to the extent that would be demanded if the large amount of originally active extract be taken into account. Lack of material prevented us from making a more careful study of this method at the time, especially with regard to the influence of temperature and the maximum strength of free acetic acid that can be tolerated by the hæmolysin. The result of the experiment nevertheless points unmistakably to the conclusion that our hæmolytic principle must be classed with the glucosides. This inference is further strengthened by the following experiment.

Dialysis and Removal of Proteid. A quantity of the highly active methyl alcohol precipitate obtained from 20 grams of the dried fungus was dialysed in the presence of thymol in a collodion sac against distilled water for three days, the sac being renewed every twelve hours. Although the dialysing solution remained free from bacteria its hæmolytic power slowly diminished. Both pigment and glucoside appeared in the dialysate. After the dialysis had continued uninterruptedly for three days the contents of the sac were reduced to a small volume at a low temperature and freed from proteid with freshly prepared metaphosphoric acid. On neutralizing the proteid-free solution it was found to be actively hæmolytic. It was now evaporated to dryness and the small residue thus obtained was subjected to qualitative analysis. Its chief constituent was a glucoside requiring previous hydrolysis with an acid for the full development

of its reducing power for Fehling's solution or ammoniacal silver nitrate. With this glucoside were associated a little pigmentary matter and the salts due to the use of metaphosphoric acid in the removal of proteids. The same results were obtained in a number of experiments with collodion sacs and we therefore believe that dialysis leads again to the conclusion that our hæmolytic principle is a glucoside.

The Dialysates. All portions of the dialysate, even when tested after being concentrated at a low temperature, were found to be quite devoid of hæmolytic activity. Chemical tests showed that the dialysate contained a glucoside which behaved toward reagents in every way like that which still remained in the dialysing sac and like that which was obtained by the use of basic lead acetate. The later portions of the dialysate contained this glucoside in *relative* excess as compared with other constituents. The small amount of active glucoside left behind after dialysing for three days cannot represent the total amount of hæmolytic substance with which we began the experiment, and we are forced to conclude that the active glucoside is so altered in its passage through the wall of the sac as to be deprived of its hæmolytic properties. Kobert found that the hæmolysin will not pass through parchment; we have not yet had the opportunity of trying this method.

REACTIONS AND PROPERTIES OF THE HÆMOLYTIC GLUCOSIDE.

We have thus been able to show that Amanita-hæmolysin cannot be classed with *proteids* or *toxalbumins* but must be considered a glucoside. We do not claim that we have freed this glucoside from all traces of foreign matter. We hope in the near future to obtain it in a state sufficiently pure for elementary analysis, without loss of its hæmolytic properties, but to secure both of these conditions at the same time may be found to be as difficult in this case as with certain other glucosides, such as the saponins.

In its present state of purity this hæmolytic glucoside shows the following reactions:

1. It reduces Fehling's solution and ammoniacal silver nitrate only very slightly without previous hydrolysis with a mineral acid, but very powerfully after such hydrolysis.

2. It gives an abundant precipitate with neutral or basic lead acetate and a precipitate with tannic acid soluble in water and in excess of tannic acid.

3. With phosphotungstic and phosphomolybdic acids it gives a faint turbidity suggesting the presence of an impurity.

4. Cupric acetate fails to precipitate the glucoside but reveals the presence of retained phosphates.

5. It does not ferment with brewer's yeast either before or after hydrolysis with acids. The small amount of fermentation gas obtained on a few occasions after eighteen hours in the thermostat was referred by us to bacterial action rather than to fermentation by yeast. A larger quantity of gas obtained after four days in the thermostat was found to consist largely (33 per cent.) of hydrogen and combustible gases, thus verifying our conclusion in regard to bacterial action in the exceptional cases above mentioned.

6. The glucoside gives the following tests for pentoses in the most satisfactory manner:

a. A fine purple violet color on heating gently with α-napthol and sulphuric acid.

b. A fine cherry red color after similar treatment with phloroglucinol and hydrochloric acid.

c. A deep green color on heating gently with orcinol, hydrochloric acid, and a drop of ferric chloride solution.[1] Like the red pigment formed in the preceding reaction, this green pigment is easily soluble in amyl alcohol.

d. It decolorizes an alkaline solution of potassium permanganate at room temperature.

e. It gives a yellow color on boiling with sodium hydrate if it be first carefully hydrolyzed with hydrochloric acid.

These tests when considered in connection with the behavior of our glucoside toward brewer's yeast, Fehling's solution, and ammoniacal silver nitrate show conclusively that the sugar contained in the molecule is a pentose. It remains for future experiments to determine which pentose or methyl pentose is present.

7. All specimens of our hæmolytic glucoside after having been freed from proteid and otherwise purified as above stated,

[1]Bial, *Deutsch. med. Wochenschr.*, 1903, p. 477.

either by dialysis or by treatment with basic lead acetate, still contain nitrogen as is proved by positive results obtained by the Laissaigne test. Such specimens also evolve alkaline vapors when heated with sodium hydrate. These vapors have an odor that is suggestive of methylamine. The fact that all specimens of our hæmolysin contain nitrogen, whether obtained by dialysis or by the use of basic lead acetate, leads us to infer that this element is an integral constituent of its molecule. The experiments that have been cited lead us to believe that our hæmolytic substance is a nitrogenous glucoside which is very sensitive toward the action of heat, acids, and certain glucoside-splitting ferments and which is easily decomposed by acids so as to yield a pentose and a volatile base or bases, such as ammonia and methylamine.

We hope in the near future to give the results of further work in this field, especially as to the nature of the thermostabile poison (or poisons), which we have called the Amanita-toxin, with which Ford has immunized animals and for which he has produced an antitoxic serum. We have not yet brought our study of this substance to a satisfactory conclusion. It is our purpose to discover if possible whether this poison bears any chemical relation to the hæmolytic glucoside above described or to the alkaloid which Kobert declares to be present in the fungus.

SUMMARY.

1. The fact that an immunity can be established toward the two poisonous principles of *Amanita phalloides* and that the serum of immunized animals is antihæmolytic and antitoxic in character gives renewed interest to a chemical study of these poisons.

2. The hæmolytic principle contained in the Amanitas and first detected by Kobert is not a toxalbumin as this author supposed, but a nitrogenous glucoside which is very sensitive toward the action of heat and acids, less so toward the action of alkalies, and is easily decomposed by acids so as to yield a pentose and a volatile base or bases, such as ammonia and methylamine.

3. This glucoside, which we designate Amanita-hæmolysin,

is present in fresh specimens of *A. phalloides* and *A. citrina*. Seibert failed to detect its presence in fresh extracts of *A. citrina* and we therefore conclude that specimens of this fungus growing in different parts of the world may or may not contain this glucoside.

4. The properties of the Amanita-hæmolysin are such as to preclude it from playing any rôle as a *blood poison* in case of poisoning by the Amanitas.

THE GLYOXYLIC ACID REACTION FOR TRYPTOPHAN, INDOL, AND SKATOL.

By H. D. Dakin.

(*From the Laboratory of Dr. C. A. Herter, New York.*)

(Received for publication, August 16, 1906.)

The reaction for the detection of proteins originally described by Adamkiewicz[1] consisted in the addition of strong sulphuric acid to the acetic acid solution of the substance under investigation. In the presence of proteins a reddish violet ring is produced at the junction of the fluids which spreads throughout the solution on standing or on gentle shaking. This test was subsequently investigated by Hopkins and Cole,[2] who found that proteins did not uniformly yield a positive reaction and that the differences were attributable to the acetic acid employed in the test. They found that *pure* acetic acid did not give the reaction and so they were led to the belief that an essential condition for the success of the Adamkiewicz reaction was the presence of some substance commonly found as an impurity in acetic acid. As is well known, they eventually concluded that this impurity was glyoxylic acid, for they found that a dilute solution of glyoxylic acid gave the reaction in a typical fashion and that the presence of acetic acid was unnecessary.

By the subsequent isolation of tryptophan from the products of protein hydrolysis, Hopkins and Cole[3] were able to show that the Adamkiewicz reaction was due to the presence of tryptophan groupings in the protein molecule, for not only did tryptophan itself show the same reaction with glyoxylic acid, but positive results were obtained with all proteins containing the tryptophan grouping but not with gelatin, which yields no tryptophan.

[1] *Arch. f. d. ges. Physiol.*, ix, p. 156, 1874; *Ber. d. deutsch. chem. Gesellsch.*, viii, p. 161, 1875.

[2] *Proc. of the Roy. Soc.*, lxviii, p. 23, 1901.

[3] *Journ. of Physiol.*, xxvii, p. 418, 1900; xxix, p. 451, 1902.

Recently, however, a paper has appeared by Rosenheim[1] in which the correctness of some of the results of Hopkins and Cole is questioned. Rosenheim shows that a typical Adamkiewicz reaction may be obtained by the addition of commercial sulphuric acid to a protein or tryptophan solution to which a minimal quantity of formaldehyde has been added. He finds, however, that pure sulphuric acid and pure formaldehyde do not yield the reaction as the presence of some oxidizing agent is necessary. The oxidizing substance may be conveniently introduced into pure sulphuric acid by the addition of traces of ferric chloride, hydrogen peroxide, or potassium persulphate, etc. Excess of formaldehyde must be avoided as under these conditions the reaction is interfered with.[2]

Rosenheim considers that the Adamkiewicz reaction as previously obtained with commercial acetic acid or with glyoxylic acid was really due to the presence of formaldehyde. He further states that he has had three samples of "pure" glyoxylic acid which gave no reaction when tested with proteins and pure sulphuric acid but it must be noted that no information is given as to the method of preparation of any of these specimens, nor is any evidence offered of their purity. A sample of glyoxylic acid prepared by the reduction of oxalic acid gave the reaction, but this Rosenheim ascribes to the presence of persulphuric acid, or hydrogen peroxide.

As the writer[3] made use of the reaction with skatol and with tryptophan, in conjunction with other tests for the detection of glyoxylic acid, it was of importance to inquire into the correctness of Rosenheim's conclusions. Although Rosenheim's results with regard to the reactions between formaldehyde, proteins or tryptophan, and sulphuric acid are confirmed, it has not been possible to obtain any evidence in support of his statement that glyoxylic acid does not yield the reaction. Experience with this reaction completely confirms the original statements of Hopkins and Cole. Glyoxylic acid or its salts have been prepared in many different ways and in all cases the products have given an

[1] *Biochem. Journ.*, i, p. 233, 1906.

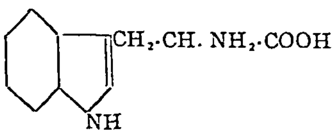
[2] The Hehner test for the detection of formaldehyde in milk and its many modifications are merely applications of the tryptophan-formaldehyde reaction. Cf. Acree, This Journal, ii, p. 145, 1906.

[3] This Journal, i, p. 271, 1906.

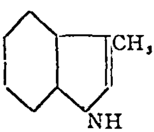
extremely delicate reaction with tryptophan (or protein) and strong sulphuric acid. The following methods of preparation have been employed:

I. The oxidation of alcohol with nitric acid.[1]
II. The reduction of oxalic acid with sodium amalgam.
III. The electrolytic reduction of oxalic acid.
IV. The action of water upon dibromacetic acid.
V. The action of water upon dichloracetic acid.
VI. The action of silver oxide upon dichloracetic acid.
VII. The action of water upon silver dichloracetate.

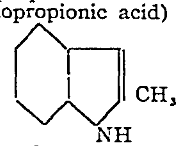
The first two methods were used by Hopkins and Cole in the preparation of their acid but Rosenheim considers that "the methods employed by Hopkins and Cole for the preparation of glyoxylic acid do not absolutely exclude the presence of oxidizing agents." This objection can, however, hardly be applied to all the other methods of preparation. Using the purest glyoxylic acid (or its salts) that I have been able to obtain, I find that the reaction with tryptophan is as sensitive as Rosenheim's formaldehyde reaction and that colored products are also obtained with skatol and indol. Skatol yields a bright pink-red coloration which does not change on dilution with water, while indol yields a somewhat darker red color, and α-methylindol also yields colored products but the reaction in this case is much less sensitive. The relationship between these substances is seen from the following formulæ:



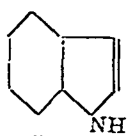
Tryptophan
(indol-aminopropionic acid)



Skatol
(β-methylindol)



α-Methylindol



Indol

Brief reference to a few points in the preparation of glyoxylic acid by the different methods may be made.

[1] Debus, *Ann. d. Chem.*, c, p. 56, 1856; Böttinger, *ibid.*, cxcviii, p. 206, 1879.

The electrolytic reduction of oxalic acid was performed in sulphuric acid solution according to Traube's directions, using a divided cell and leaden electrodes.[1] It is most important that rise in temperature inside the cathode chamber be avoided as otherwise glycollic acid forms the main product of reaction. At the end of the reduction, when no more oxalic acid was present, the liquid was diluted, exactly freed from sulphuric acid by barium hydroxide, and the free glyoxylic acid was then converted into the calcium salt which was purified by repeated crystallization from water. The product in every case gave a typical reaction with tryptophan, indol, and skatol in extremely dilute solution.

Dibromacetic acid was prepared by the direct bromination of pure acetic acid.[2] The product was fractionated under ordinary pressure and the portion boiling at 230–235° C. was used. After simply boiling with water or better by heating in a sealed tube with water at 135° C.,[3] the solution gave an intense reaction with tryptophan and strong sulphuric acid.

$$\mathrm{CHBr_2COOH + H_2O = \underset{\displaystyle H}{\overset{\displaystyle O}{\overset{/\!/}{C}}} - COOH + 2HBr}$$

The action of silver oxide upon dichloracetic acid has been investigated by Beckurts and Otto.[4] Two molecular proportions of pure dichloracetic acid (*i.e.* an excess) which had been prepared by the action of potassium cyanide upon chloral[5] were boiled with one molecular proportion of freshly precipitated silver oxide, suspended in water. Decomposition takes place in accordance with the following equation:

$$\mathrm{CHCl_2 \cdot COOH + Ag_2O = \underset{\displaystyle H}{\overset{\displaystyle O}{\overset{/\!/}{C}}} - COOH + 2AgCl}$$

[1] Traube, *Ber. d. deutsch. chem. Gesellsch.*; *cf.* also D.R.P., 163,842 (*Chem. Centralbl.*, ii, p. 1699, 1905).

[2] Perkin and Duppa, *Ann. d. Chem.*, cx, p. 115, 1859.

[3] Grimaux, *Bull. de la soc. chim.* (Paris), xxvi, p. 483, 1876.

[4] *Ber. d. deutsch. chem. Gesellsch.*, xiv, p. 585, 1881.

[5] Chloral itself gives no reaction with tryptophan and strong sulphuric acid.

The silver chloride was filtered off and a trace of the filtrate was found to react in a typical manner with tryptophan and sulphuric acid. In order to separate the glyoxylic acid from the excess of dichloracetic acid, the acids were converted into their calcium salts by means of calcium carbonate. On concentration, well-formed crystals of calcium glyoxylate crystallized out and were separated from the more soluble calcium dichloracetate. The calcium salt was twice recrystallized from water and was then tested in the following way:

Reaction with Tryptophan. A solution of pure crystallized tryptophan was used, containing one-tenth of a milligram in one cubic centimeter. One cubic centimeter of this solution was added to one cubic centimeter of calcium glyoxylate solution and then two to two and one-half cubic centimeters of pure sulphuric acid were added. The color at the contact zone was first observed and then the liquids were caused to slowly mix by gentle shaking. In a second series of experiments, one cubic centimeter of a calcium glyoxylate solution containing one-tenth of a milligram was used and the amount of tryptophan was varied. The results were as follows:

Calcium Glyoxylate. Grams.	Tryptophan. Grams.	Result.
0.001	0.0001	Intense reaction.
0.0001	"	Strong reaction.
0.00001	"	Slight reaction.
0.000005	"	Slight but positive reaction.
0.000001	"	No result.
0.0001	0.00005	Strong reaction.
"	0.00001	Slight but positive.
"	0.000005	Just positive.

The results show that calcium glyoxylate prepared in the way described reacts in a typical manner with one-tenth of a milligram of tryptophan at a dilution of one part in two hundred thousand and conversely using one-tenth of a milligram of calcium glyoxylate, one part of tryptophan in two hundred thousand parts of water may be detected.

The same specimen of calcium glyoxylate was tested with indol and with skatol. The sensitiveness of the test with indol is approximately the same as that with tryptophan, but with

skatol the reaction is still perceptible at a slightly greater dilution.

Calcium Glyoxylate. Grams.	Skatol. Grams.	Result.
0.0001	0.0001	Intense pink-red solution.
0.00001	"	Strong reaction.
0.000005	"	Very distinct.
0.000001	"	Easily perceptible.
0.0001	0.00001	Very strong reaction.
"	0.000005	Strong reaction.
"	0.000001	Slight but positive.

Entirely similar results were obtained with glyoxylic acid prepared by the action of water upon silver dichloracetate.[1] Dichloracetic acid was converted into the neutral sodium salt and then precipitated with an equimolecular proportion of silver nitrate. The precipitated silver salt was filtered off with the pump and washed with ice-cold water. It was then scraped into a flask and boiled with water under a reflux condenser. The salt decomposes with formation of glyoxylic acid and dichloracetic acid, which were separated as before. The products reacted in the usual way with tryptophan, indol, and skatol.

$$2CHCl_2{\cdot}COOAg + H_2O = \overset{\displaystyle O}{\underset{\displaystyle H}{\overset{/\!/}{\underset{\diagdown}{C}}}}{-}COOH + CHCl_2.COOH + 2AgCl$$

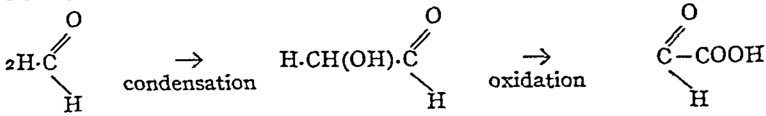
It is difficult to believe that these uniformly positive results are due to impurities[2] and it is therefore concluded that, contrary to the statement of Rosenheim, pure glyoxylic acid reacts with tryptophan and pure sulphuric acid to give the characteristic color reaction.

The products obtained when glyoxylic acid and formaldehyde and oxidizing substances act upon tryptophan and skatol are similar and possibly identical and it is not inconceivable that they are both due to the action of the same substance. Since glyoxylic acid alone gives the reaction, while formaldehyde needs the presence of oxidizing substances, it may be conjectured that

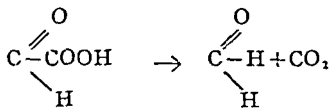
[1] Beckurts and Otto, *loc. cit.*

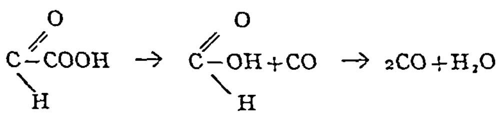
[2] The results are not influenced by boiling the solutions of calcium gyloxylate for some time or by heating the dry salt for an hour at 150° C.

the formaldehyde is converted into glyoxylic acid. If formaldehyde, like acetaldehyde, were to undergo the aldol condensation under the influence of acids, its conversion by oxidation into glyoxylic acid could be readily accounted for.



With the view of testing this possibility, formaldehyde was oxidized with hydrogen peroxide in the presence of strong sulphuric acid, but the formation of glyoxylic acid could not be satisfactorily demonstrated, although the experiments made do not entirely preclude the possibility of its formation. On the other hand it is possible that the glyoxylic acid may undergo decomposition yielding the same products as are obtained from the formaldehyde. Theoretically at least it may be converted into formaldehyde by loss of carbon dioxide, while on the other hand the action of strong sulphuric acid is known to remove carbonic oxide from many acids containing two carbonyl groupings,[1] so that the production of carbonic oxide and formic acid or simply carbonic oxide and water might be considered.





But neither formic acid nor carbonic oxide, either alone or in conjunction with formaldehyde, react in a typical manner with tryptophan, so that the experimental evidence is at present insufficient to decide whether the chromogenic substance in the case of the glyoxylic and formaldehyde reactions is the same.

Reference may be made to the difference in the action of formaldehyde and glyoxylic acid upon indol in the presence of sulphuric acid. Glyoxylic acid, as has already been mentioned,

[1] Bistrzycki and B. v. Siemiradzki, *Ber. d. deutsch. chem. Gesellsch.*, xxxix, p. 51, 1906.

gives a red coloration, whereas formaldehyde gives a violet-blue color very similar in tint to that obtained with glyoxylic acid and tryptophan but which differs from the latter on account of its permanence on dilution with water. This reaction, which has recently been described by Konto,[1] differs in other respects from the formaldehyde reaction with tryptophan. Apparently no oxidizing agent is needed as specimens of formaldehyde and pure sulphuric acid which gave no reaction with tryptophan unless a trace of ferric chloride or some other oxidizing substance were added, reacted directly with indol. A further distinction is found in the fact that the reaction with indol is not abolished by a slight excess of formaldehyde, while the tryptophan reaction is not obtained under these conditions. The sensitiveness of the two reactions is approximately the same. Skatol under similar conditions with formaldehyde and pure sulphuric acid gives an ill-defined brownish color, but if a trace of an oxidizing agent be added to the sulphuric acid, the characteristic reddish-pink coloration is obtained.

The preceding experiments have necessarily in some degree trenched upon the line of investigation followed by Dr. F. G. Hopkins and it is with his kind consent that the foregoing observations are published.

[1] *Zeitschr. f. physiol. Chem.*, xlviii, p. 185, 1906.

CASES OF DIABETES TREATED WITH SECRETIN

BY NELLIS B. FOSTER.

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(Received for publication, August 15, 1906.)

INTRODUCTION.

The methods of treatment of diabetes mellitus are so inadequate and in the adolescent cases usually so hopeless that any manner of therapeutics that is not obviously absurd demands careful consideration and systematic trial. The observations of Bayliss and Starling[1] on the stimulating effect produced on the secreting power of the pancreas by injecting into animals an acid extract of the duodenal mucous membrane suggested the idea to Spriggs[2] that the effect of secretin, as the active agent in duodenal extract is called, might not be confined to the external pancreatic secretion but possibly might also be exerted on the *internal* secretion. If it have any such power then it may be of use in some cases of diabetes, such, for example, as are due to disordered pancreatic function where there is, perhaps, early in the course of the disease, no pronounced organic change, especially in cases in which the excitant (secretin) might be lacking for one reason or another. The result of Spriggs' observations were negative. Working on the same idea Moore[3] and his associates studied three cases of diabetes mellitus treated with secretin. Two of these cases ceased to have any glycosuria after a month's treatment. The cases of apparent cure were children who usually, as every one knows, quickly succumb to this malady. One case was quite uninfluenced by the course of the treatment. One of the patients who was apparently cured has

[1] Bayliss and Starling, *Proc. of the Roy. Soc.*, lxix, p. 352, 1902; *Journ. of Physiol.*, xxviii, p. 325, 1902; xxix, p. 174, 1903.

[2] Cited by Moore, *Bio-Chem. Journ.*, i, p. 30, 1906.

[3] *Loc. cit.*, p. 28.

since the publication of Moore's paper, relapsed (personal communication). All of Moore's cases had been on a carbohydrate-free diet for a short period (less than a month) preceding the course of secretin-treatment, but this period was not sufficiently long to insure that no further diminution in the output of urinary glucose was possible for those diabetics under the prevailing conditions. It has been my experience that not infrequently months of the most careful experimentation with diets are necessary to derive for any patient the full benefit of a simple dietary of meats, fats, and green vegetables. The results of Moore's work were so promising, however, that, at the suggestion of Dr. Gies, I repeated the experiments in this laboratory, but up to this time I have had no case which showed in any degree beneficial results following the use of secretin.

EXPERIMENTAL.

The cases of diabetes here reported were patients in Professor Lambert's clinic at Vanderbilt Clinic, one of Professor Lambert's patients in private practice, and two patients of my own. Three of the cases had been under constant observation for over one year and the effects of various diets carefully noted. Those cases of diabetes who were seen for the first time while these studies were in progress were subjected to the following regimen: (*a*) A period of unrestricted diet to ascertain the quantity of sugar ordinarily excreted, (*b*) a period of carbohydrate-free diet when only meat, fats, and sugar-free green vegetables were allowed, and (*c*) a period during which was determined the effect on the urinary glucose of small amounts of such carbohydrate foods as bread, potato, or milk; each of these three additions to the diet being tried separately for several days. The estimation of sugar was made only on twenty-four hour specimens of urine. After a sufficient time had elapsed to give me a fair idea of the condition of the patient the active treatment with secretin was commenced.

Preparation of Secretin Extract. The extract of the duodenal mucous membrane was prepared in accordance with Moore's directions. I used pig intestines, which were removed from the animal immediately after slaughter and taken at once from the abattoir to the laboratory. About two feet of each duodenum

was separated for use. Such sections from about twenty-five intestines were extracted at a time. The gut was opened, quickly washed with physiological salt solution, and the mucosa lightly scraped off with a horn spatula. After passing the mucous membrane through a fine hashing machine it was mixed with an equal volume of 0.4 per cent. hydrochloric acid, ground in a mortar, then transferred to a casserole and slowly heated to boiling. Filtration through cheese-cloth freed the material from the larger particles of membrane. After rendering the filtrate less acid by adding sodium hydroxide until the reaction to litmus was faintly though still distinctly acid, it was sterilized in flasks and was ready for use. This secretin extract was freshly prepared every three days. The dose I have employed was an ounce of this extract a half hour after meals.

CLINICAL HISTORIES.

CASE I. Charles M., age 60, has known for four years that he had diabetes, but with the exception of occasional mild attacks of sciatica, he has experienced little inconvenience from his disease. He came under my observation in 1904 when he had been losing weight rather rapidly; at that time he was voiding an average of 2500 c.c. of urine daily and the glucose elimination fluctuated between 50 and 60 grams per day. He had been observing some care as to his diet. A decrease of the carbohydrate in the food produced no result on the output of urinary sugar but improved the patient's condition to some extent and his weight increased several pounds. Various diets and drugs were used without any decided result, and beginning in April, 1906, he was given the secretin extract for five weeks, in doses of one ounce after meals. There was no marked change in the amount of sugar excreted during the time of treatment, the last analysis made during the period showing 57.5 grams of sugar for the 24-hour specimen of urine; nor was there during the period of treatment or since its discontinuance any improvement in the general health of this patient. The following table shows the urinary condition during the period of treatment with secretin.

	24-Hour Urine.	Sugar.
April 16	2350 c.c.	51.0 grams
19	2280 "	49.6 "
21	2470 "	55.2 "
23	2515 "	54.8 "
25	2490 "	56.2 "
28	2525 "	58.1 "
30	2180 "	45.7 "

	24-Hour Urine.	Sugar.
May 2	2460 c.c.	62.9 grams
5	2015 "	60.1 "
7	2360 "	61.6 "
9	2140 "	60.0 "
11	2440 "	59.2 "
14	2475 "	60.7 "
16	2340 "	57.5 "
18	2410 "	56.3 "
21	2370 "	55.7 "
23	2315 "	52.1 "
25	2430 "	54.3 "
28	2490 "	57.5 "

Case II. Richard E., age 48, a well nourished, strong looking man, was first seen at the Vanderbilt Clinic in February, 1906. He said he had known of his glycosuria for about a year. He had been advised to avoid sugar and starches in his diet and he says he has observed the precaution, but the glucose has since never been below 3 per cent. in his urine and of late he has lost some 30 lbs. in weight. He was advised to use a rigid carbohydrate-free diet, which diminished the glycosuria to some extent but was not effectual in reducing the output below 60 grams per day. After a month's observation on the effect of diet the secretin extract was given and urinary analyses were made every other day for 29 days. The urinary sugar at once began to diminish and in the course of 3 weeks the daily amount had fallen to 31 grams for the 24 hours. The patient had, however, continued to lose in weight and had become so weak he was unable to go to the clinic. The urine analyses were so encouraging that the treatment was persisted in nevertheless for another week, but during this time the daily output of sugar began to increase again. On the 29th day the amount was 37.9 grams and the condition of the patient so bad that this line of therapeutics was abandoned. He was confined to his bed for two weeks, unable to eat any solid food because of a nauseating, sweet taste, and complained of a most profound feeling of weakness. He had no fever at any time. At the end of three weeks he was again able to be up, and rapidly regained his appetite and strength. Since the course of treatment the urine has shown approximately the same content of sugar as before. He is, however, stronger and has gained 5 lbs. in weight. Whether this was due to the influence of secretin, and the question as to the nature of the intervening sickness, I am unable to answer. The analyses of the urine at that time gave no indication of coma, nor was the condition of the patient suggestive of that complication.

Case III. Mrs. R. O., age 65, has been treated 6 years for diabetes. During the year of 1905 she dieted to a moderate degree and the urine reports for this period show a considerable variation in the daily sugar elimination between 77 and 154 grams, the average being about 105

grams. In April, 1906, the diet was considerably restricted but not all starch foods were interdicted; there was, however, no effect on the glycosuria. Following this month of observation secretin extract was given for a month without result so far as could be noted.

CASE IV. Mary B., age 44, had been under treatment at Vanderbilt Clinic for 14 months. When she first came under my observation her symptoms were the usual ones of mild diabetes; thirst and polyuria were not marked. She is a large, stout, German woman without any sign of organic disease other than the glycosuria. Her condition improved considerably during the year after her diet had been restricted, but the urinary sugar has remained quite constant, between 30 and 40 grams daily. In May of this year she was given secretin for 3 weeks after which it was discontinued on account of a strong aversion to the taste of the material, which on several occasions caused vomiting. There was no perceptible change in the glycosuria during or following this period.

CASE V. Robert N., age 60. Was rejected for life insurance in January, 1906, on account of glycosuria. He is a large, well preserved man of healthy aspect. He had considered himself in perfect health until the above incident. He has had absolutely no symptoms and a careful physical examination gave no evidence of disease. An examination of collections of 24-hour urine during 2 weeks of unrestricted diet showed a daily elimination of 70 to 100 grams of glucose. This was reduced to an average of 50 grams by means of a rigid carbohydrate-free diet for 3 weeks. Secretin extract was then given for a month but without any notable change resulting either in the patient's condition, which was excellent, or in the degree of glycosuria. Following the discontinuance of the use of the secretin, the amount of food permitted was reduced so that the total per diem would not exceed 1800 calories. This, with the interpolation of one fast day each week when green vegetables and buttermilk only were advised, resulted in reducing the urinary sugar to an average of 20 grams per diem, and an increase in body weight from 196 to 198 lbs.

In addition to these five cases secretin extract has been used with four other cases of diabetes without favorable result. Inasmuch as circumstances compelled me to entrust these four patients entirely with the carrying out of directions regarding diet, the use of medicines, and the collection of urine for analysis, I have no certainty of the data and will not present them.

CONCLUSIONS.

In view of these results I am unable to confirm the observations of Moore and his associates. The apparent failure of secretin to affect these cases of diabetes is in accord with Spriggs's observations. But these negative results do not exclude the

possibility that there are cases which might respond to this method of treatment. Concerning the pathology of diabetes we are still much in the dark; morbid change in the pancreas that can be detected with the means at present at our disposal is absent in a large percentage of cases, and even the significance of what has been held to be a pathological process in the islands of Langerhans has been questioned. In the cases where there are organic changes in the pancreas it is not to be expected that a stimulating agent like this secretin extract would have any effect. But as Moore has suggested, there are two other possible causes which may give rise to glycosuria, namely, (*a*) the non-secretion of the pancreatic excitant by the duodenum; (*b*) the duodenum being normal, and the pancreas supplying its internal secretion, there may be a perverted function on the part of the oxidizing organs, such as the liver and muscles. There is a pathological basis for the last suggestion in the association of diabetes with cirrhosis of the liver in certain cases. Of these three possible conditions which may cause glycosuria, in only one would the secretin extract be of any service as a therapeutic agent, namely, when the function of the duodenal cells is so disturbed that insufficient pancreatic excitant is secreted.

If such a condition as Moore suggests exists it can at present only be detected by a systematic use of this secretin extract. It will require much more extended observation than has yet been made to estimate the utility, if any, of this therapeutic agent.

I desire to express my obligation to Prof. Samuel W. Lambert for opportunity to conduct these studies and for numerous valuable suggestions.

ADDENDUM.

After the first proof of this paper had been read, my attention was called to a paper by Bainbridge and Beddard in the September number of *The Bio-Chemical Journal* (Vol. I, Nos. 8 and 9, pp. 429–446). After using secretin in the treatment of three cases of diabetes those authors conclude: "The administration of secretin by the mouth had no influence whatever upon the output of sugar in the urine."

In some further investigations on prosecretin, Bainbridge and

Beddard find that only in one out of six cases of severe diabetes was prosecretin present in an amount approximating to the normal. This observation suggests that in certain diabetics secretin may play an important part in the etiology of this disease, and likewise calls up many important questions for solution.

NOTE ON THE TREATMENT OF A CASE OF DIABETES MELLITUS WITH SECRETIN.

By H. D. DAKIN and C. C. RANSOM.

(*From the Laboratory of Dr. C. A. Herter, New York.*)

(Received for publication, October 20, 1906.)

The announcement by Moore[1] of the successful treatment of certain cases of diabetes by means of the acid extract of duodenal mucous membrane (secretin) appeared to be of such importance that it seemed desirable to repeat the experiments and if possible to obtain confirmatory results.

It will be recalled that the indications for the use of secretin depend upon the known relationships existing between many cases of that disease and the pancreas, for it is clear that a substance such as secretin, which calls forth the external pancreatic secretion, might exert a similar effect upon any internal secretion and so indirectly exert an influence upon the sugar output. If the preceding considerations have any foundation in fact, the most favorable opportunity for observing the influence of secretin upon diabetes would be afforded by a patient with a past history of pancreatic disorder. The present note consists of a brief record of the observations upon a single case of this kind, and although further trials are desirable the results may be compared with those of Dr. Foster, recorded in the preceding paper.

The patient was a married woman, forty-five years old, who for several years had experienced intermittent attacks of severe pain in the epigastric region. There was present a well-defined and easily palpated mass in the region of the pancreas. After other possibilities had been duly considered, the condition was

[1] B. Moore, *Biochem. Journ.*, i, p. 28, 1906.

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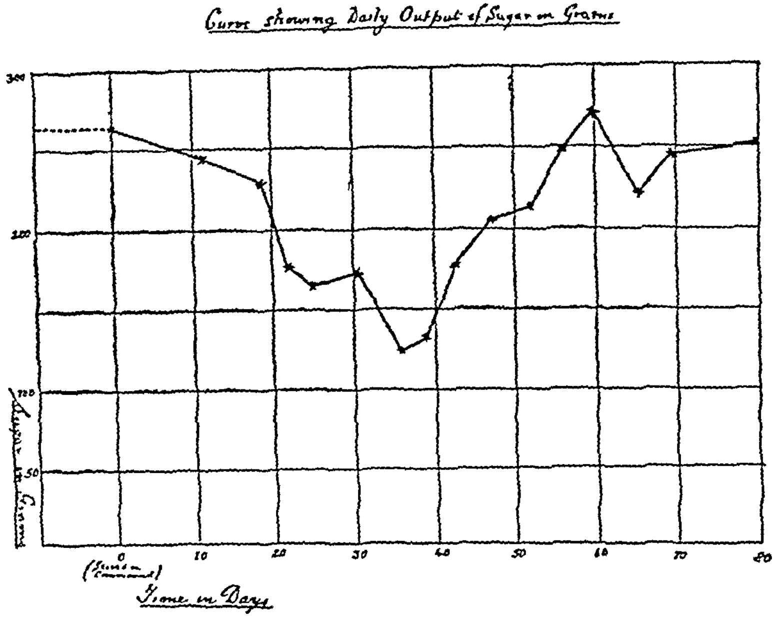
confidently diagnosed as pancreatic disease. These seizures had caused great loss of weight and the patient was generally in poor condition, but subsequently a great improvement took place and at the time that treatment with secretin was commenced she appeared fairly healthy and well nourished. About four years ago a large amount of sugar was detected in the urine, which could not be materially reduced by dieting, and for some time before the treatment commenced the patient was in the habit of daily excreting about three liters of urine containing seven to eight per cent. of sugar. During the whole time of treatment with secretin, and for some months previously, the patient was on a restricted but not carbohydrate-free diet and no drugs were used. The secretin was freshly prepared three times a week from the upper six feet of the small intestines of freshly slaughtered pigs. The details of preparation were essentially those adopted by Bayliss and Starling and by Moore. The use of secretin was maintained during twelve weeks, during which period the extract from about four hundred feet of intestine was consumed.

The percentage of sugar in the urine at different periods is given in the following table, while the daily output of sugar may be followed in the accompanying curve:

Time of Treatment.	Sugar.
0 days	8.0 per cent.
1 day	8.0 "
11 days	8.1 "
17 "	8.3 "
22 "	7.0 "
25 "	6.6 "
32 "	6.5 "
35 "	4.2 "
38 "	4.2 "
42 "	5.0 "
46 "	5.3 "
53 "	6.0 "
57 "	7.1 "
60 "	7.7 "
66 "	6.4 "
69 "	7.0 "
80 "	7.3 "



It will be seen that there was but little change in the sugar excretion during the first fortnight, but in the third week a distinct decrease was observed, and this decrease in sugar, which was accompanied by a diminished urine output, was continued until, after five weeks, the original sugar excretion was reduced by one half. At this point the patient had a seizure of acute pain, which was referred to the pancreas and which differed from the previous attacks only in its increased severity.

After a few days the pain disappeared but the sugar excretion gradually rose, despite the continued use of secretin in increased doses, until the original output of sugar was practically reached. During the latter part of the period of observation the urine contained large amounts of acetoacetic acid and acetone.

The results appear to show that, though it is probable that the use of secretin resulted in a diminished sugar excretion, the diminution was not permanent nor was it nearly so great as in the cases described by Moore.

THE CHEMISTRY OF FLESH.

(Fifth paper.)[1]

METHODS FOR THE DETERMINATION OF CREATININ AND CREATIN IN MEATS AND THEIR PRODUCTS.

By H. S. GRINDLEY and H. S. WOODS.

(From the Chemical Laboratory of the University of Illinois.)

(Received for publication October 26, 1906.)

In the previous papers from this laboratory it has been conclusively proven by improved methods of analysis that meats contain a considerably greater quantity of so-called organic extractives than previous investigations had shown. It has also been demonstrated that a considerable proportion of flesh is soluble in cold water; that the cold water extraction of meat can be made complete and that it is possible to determine with accuracy the total nitrogenous organic extractives of such extracts. In connection with the researches of this laboratory upon the chemistry of flesh it became desirable to devise means to ascertain the relative proportion of the different components which go to make up these extractives. The creatin and also the creatinin if present in meats would undoubtedly be completely extracted by cold water. That being true, an attempt was made to apply the colorimetric method of Folin[2] to the estimation of the creatinin and creatin, using the Duboscq colorimeter.

Tests for Creatinin in Meats.

In the first place the method was used to test for creatinin in a cold water extract of chicken flesh. Two hundred cubic centimeter portions of the extract representing 4.112 grams of fresh

[1] *Journ. Amer. Chem. Soc.*, xxvi, p. 1086, 1904; xxvii, p. 658, 1905; xxviii, pp. 25 and 469, 1906.

[2] *Zeitschr. f. physiol. Chem.*, xli, p. 223, 1904; *Amer. Journ. of Physiol.* xiii, p. 48, 1905.

chicken flesh were tested directly for creatinin in the manner described by Folin in estimating this constituent in urines. The tests gave only the slightest indications, if any, of the presence of creatinin. When the alkaline picrate solution of the extract of the chicken flesh was allowed to stand for a few days, it turned red, the color growing more intense each day. Water extracts of beef and solutions of meat extracts when treated directly with the alkaline picrate solution behaved in a similar manner, showing that creatin under these conditions is gradually converted into creatinin even in alkaline solutions. This is contrary to the supposed behavior of these two nitrogenous bodies in the presence of alkaline solutions. The true nature of this reaction is now being further studied in this laboratory.

In order to prove further the presence or absence of creatinin in aqueous extracts of fresh flesh, 400 c.c. of a cold water extract of beef, representing all of the soluble constituents from 8 grams of lean beef round, were tested directly for creatinin as above by Folin's method. The tests gave no trace of creatinin. The alkaline picrate solutions of these beef extracts upon standing for a few hours gradually turned red, as did the extracts from chicken flesh. These experiments, which have often been repeated, demonstrate that the work and conclusions of Toppelius and Pommerehne,[1] and later that of Emil Wörner,[2] were correct, and that the investigations of G. S. Johnson,[3] which led him to the deductions that perfectly fresh meats contain creatinin, but no creatin, were wrong. As judged by Jaffe's reaction fresh meats contain only the slightest trace of creatinin if any at all. However, the creatin of flesh is in part at least very readily changed to creatinin. The following experiment demonstrates this fact. Three 500 c.c. portions of a cold water extract, each representing 10.015 grams of chicken flesh, were carefully evaporated upon a water-bath to a volume of 50 c.c. in order to completely coagulate the soluble proteid matter. The solutions were filtered, the precipitates thoroughly washed, and the filtrates evaporated to a volume of 75 c.c. They were then tested for

1 *Arch. de pharm.*, ccxxxiv, p. 380, 1896.
2 *Zeitschr. f. physiol. Chem.*, xxvii, p. 1, 1899.
3 *Proc. of the Roy. Soc.*, l, p. 287, 1901.

creatinin and they gave readings which represented 0.08, 0.11, and 0.15 per cent. of creatin, calculated upon the basis of the fresh substance of the flesh. This experiment and a number of others which we have made prove that the evaporation of an aqueous extract of flesh upon the water-bath, *in the presence* of its natural acidity, changes creatin to creatinin. It is just possible that this action may be one influence which produces flavor during the cooking of meats. *This subject is now being* studied in connection with these researches.

Estimation of Creatin in Meats.

The estimations of creatin in the aqueous extracts of flesh were made by taking 500 c.c. portions of the solution, evaporating and filtering as mentioned above to remove the coagulable proteids. The solutions thus obtained were treated with hydrochloric acid and evaporated upon the water-bath to a small volume. This treatment was repeated with the addition of water until all the creatin had been changed to creatinin. The solutions were then made up to exactly 100 c.c. and 30, 40, and 50 c.c. of the same were used to make the quantitative test with the colorimeter.

Table No. I gives a few determinations of creatin in different kinds of flesh.

TABLE I. CREATIN IN FRESH FLESH.

No. of Sample.	Kind of Meat.	Weight of Sample.	Reading of Colori-meter.	Weight of Creatinin due to Creatin. (A)	Weight of Creatin (A×1.16)	Creatin.
		Gr.	Mm.	Mgr.	Mgr.	Per cent.
2075	Beef Round	2.8066	8.2	9.88	11.46	0.41
2084	Cold Storage Fish	3.8369	7.9	10.25	11.89	0.31
2085	Cold Storage Fish	4.6625	7.8	10.38	12.04	0.26
2110	Refrigerated Chicken	4.0084	8.1	10.00	11.60	0.29
2111	Refrigerated Chicken	4.9152	8.4	9.64	11.18	0.23
2112	Refrigerated Chicken	5.0262	7.8	10.38	12.04	0.24

As a result of the preliminary experiments here reported, the following method is recommended for the estimation of creatin in flesh. Prepare the aqueous extract of the flesh as described in a former paper[1] from this laboratory.

Evaporate 500 c.c. portions of the extracts to 50 c.c., filter, wash the coagulated proteid thoroughly with hot water, add 25 c.c. of $\frac{N}{10}$ hydrochloric acid to each of the filtrates, and evaporate them to a volume of 10 to 15 c.c. Now add 50 c.c. of water and 10 c.c. of $\frac{N}{10}$ hydrochloric acid and again evaporate *to a volume of 10 to 15 c.c. Care must be taken to insure the* complete change of creatin to creatinin. Cool the solutions, transfer them to a 100 c.c. measuring flask, and dilute to the mark. Transfer an aliquot portion of this latter solution, equivalent to 200 c.c. of the original extract, to a 500 c.c. flask, add 15 c.c. of a 1.2 per cent. (saturated) picric acid solution and 5 c.c. of a 10 per cent. sodium hydroxide solution, shake well and allow the solution to stand for five minutes. In the meantime practice reading the colorimeter, using an $\frac{N}{2}$ solution of potassium bichromate in each cylinder. Now dilute the alkaline *creatinin picrate solution to 500 c.c., mix thoroughly, rinse one* of the cylinders well with this unknown solution, add a portion of the solution to the cylinder, and make several careful readings at once, comparing the unknown solution with 8.0 mm. of the bichromate solution. According to Folin, the correct reading *in millimeters divided into 81 gives the number of milligrams of* creatinin present. This figure multiplied by 1.16 gives the number of milligrams of creatin. If an aliquot portion equivalent to 200 c.c. of the extract does not give a satisfactory reading, use such a portion as will give a reading of about 8 mm.

This method has been used successfully for the determination of creatinin and creatin in uncooked and cooked meats and in meat broths and meat drippings.

Creatinin and Creatin of Commercial Beef Extracts.[2]

The nature of a considerable proportion of the constituents of

[1] *Journ. Amer. Chem. Soc.* xxvii, p. 658, 1905.

[2] After the data herein recorded had been obtained, and this paper had been written, the October number of the *Journal of the American Chemical Society* was received, containing the paper of Bigelow and Cook,

commercial beef extracts is still unknown. A ready method for the estimation of creatinin and creatin in these products would undoubtedly be useful in determining the commercial value and the grade of the various commercial beef extracts and similar preparations which are now upon the market. As Folin's method for the estimation of these substances in urines proved to be an excellent means for the determination of these nitrogenous constituents which are contained in aqueous extracts of flesh, we undertook to apply it to the determination of these bodies in commercial extracts. The method has proved equally successful in this connection. Its application here has demonstrated that commercial beef extracts contain both creatinin and creatin. The details of the procedure in the case of the extracts are entirely similar to those outlined above for cold water extracts of flesh. The results of a number of determinations of creatinin and creatin in a number of the prominent commercial extracts now on the markct are given in the following tables.

TABLE II. CREATININ IN COMMERCIAL BEEF EXTRACTS

No. of Sample.	Weight of Sample.	Reading of Colorimeter.	Creatinin.	
			Weight.	Per cent.
	Gr.	Mm.	Mgr.	
1	0.3008	7.8	10.38	3.45
2	0.2545	8.6	9.42	3.70
3	0.5800	8.1	10.00	1.72
4	0.7221	8.0	10.13	1.40
5	0.1972	7.8	10.38	5.27
6	0.7234	8.2	9.88	1.37
7	0.8206	8.2	9 88	1.20
8	0.5458	7.7	10.52	1.93
9	1.1391	8.6	9.42	0.83
10	0.4720	8.1	10.00	2.12
11	0.6786	8.5	9.47	1.39
12	0.7540	7.5	10.08	1.30
13	0.2774	7.3	11.10	4.00

upon "The Separation of Proteoses and Peptones from the Simpler Amino Bodies." In this paper, the above authors mention the fact that they have applied the method of Folin for the determination of creatin in meat extracts, but they give no analytical results. Notwithstanding this preliminary notice of Bigelow and Cook, it was deemed advisable

TABLE III. CREATIN IN COMMERCIAL BEEF EXTRACTS.

No. of Sample	Weight of Sample.	Reading of Colori-meter.	Weight of Original Creatinin plus Crea-tinin due to Creatin.	Weight of Original Creatinin (See Table I.)	Weight of Creatinin due to Creatin (A)	Weight of Creatin A × 1.16	Creatin.
	Gr.	Mm.	Mgr.	Mgr.	Mgr.	Mgr.	Per cent.
2	0.1833	7.9	10.25	6.78	3.47	4.03	2.18
3	0.2636	7.7	10.52	4.55	5.97	6.93	2.63
4	0.1850	7.9	10.25	2.60	7.65	8.74	4.79
5	0.1512	8.4	9.64	7.96	1.68	1.95	1.29
6	0.1841	8.6	9.42	2.38	7.04	8.17	4.35
7	0.1641	8.0	10.13	1.98	8.15	9.45	1.20
8	0.2593	8.0	10.13	5.00	5.13	5.95	2.29
9	0.7088	8.8	9.20	5.86	3.34	3.87	0.55
11	0.3016	5.0	16.20	4.19	12.01	13.93	4.62
12	0.2262	6.85	11.82	2.94	8.88	10.30	4.55
13	0.2080	7.00	11.57	8.32	3.25	3.77	1.81

TABLE IV. COMBINED CREATININ AND CREATIN IN COMMERCIAL BEEF EXTRACTS.

No. of Sample.	Creatinin.	Creatin.	Sum of Creatinin and Creatin.
	Per cent.	Per cent.	Per cent.
2	3.70	2.18	5.88
3	1.72	2.63	4.35
4	1.40	4.79	6.19
5	5.27	1.29	6.56
6	1.37	4.35	5.72
7	1.20	1.20	2.40
8	1.93	2.29	4.22
9	0.83	0.55	1.38
11	1.39	4.62	6.01
12	1.30	4.55	5.85
13	4.00	1.81	5.81

These results show that there are marked differences in commercial meat extracts as to their content of creatinin and creatin. In some of the extracts, creatinin occurs in considerably

to publish our paper in full and without any modifications, since it gives data which prove without doubt that the method of Folin may be applied successfully to the estimation of creatinin and creatin in meats, in meat broths, in meat extracts, and in other meat products. We have been using this method continuously in our investigations upon the chemistry of flesh since March 15, 1906.

greater quantities than does creatin, while in others the amounts of the latter constituent are greater than the amounts of the former. It is difficult to state at present the cause or causes which, in the preparation or keeping of the commercial extracts, produce this variation in the proportion of these two nitrogenous constituents. Experiments upon meat extracts prepared upon a small scale in the laboratory, from fresh meat, are now under way to find out if possible the conditions which produce the above difference in the relative proportion of creatinin and creatin.

The data here given also show that there is a marked difference in the total amount of creatinin and creatin contained in different commercial extracts now upon the market. Further study will probably show that this difference in the combined amount of these nitrogenous constituents is due, in the main, to the material from which they are manufactured. We hope to be able to study this phase of the work in the near future.

We wish here to express our thanks to Mr. H. H. Mitchell for his valuable assistance in connection with the analytical work involved in this paper.

STUDIES IN THE CHEMISTRY OF THE ION-PROTEID COMPOUNDS.[1]

IV.—ON SOME CHEMICAL PROPERTIES OF CASEIN AND THEIR POSSIBLE RELATION TO THE CHEMICAL BEHAVIOR OF OTHER PROTEIN BODIES, WITH ESPECIAL REFERENCE TO HYDROLYSIS OF CASEIN BY TRYPSIN.

By T. BRAILSFORD ROBERTSON.

(From the Rudolf Spreckels Physiological Laboratory of the University of California.)

(Received for publication, October 26, 1906.)

I. INTRODUCTION.

(a) *The Ion-Proteid Theory.*

In 1893 Dreser[2] pointed out that the greater the concentration of free mercury ions in a solution of a mercury salt the more toxic is the solution and, moreover, that the formation of complex ions containing mercury diminished the toxicity of the solution. In 1895 Klemm[3] showed that the disintegrating actions of different strong acids upon the protoplasm of cells were identical and he attributed the disintegration to the chemical action of their ions. This observation of Klemm's was followed in 1896 by the papers of Kahlenberg and True[4] and of Heald[5] showing that equivalent concentrations of strong acids, and also, in Heald's investigations, equivalent concentrations

[1] Numbers I, II, and III of these studies appeared respectively in Pfluger's *Arch. f. d. ges. Physiol.*, cx, p. 610, 1905; this Journal, i, p. 279, 1906; this Journal, i, p. 507, 1906.

[2] Dreser, *Arch. f. exper. Path. u. Pharm.*, xxxii, p. 456.

[3] Klemm, *Jahrb. f. wiss. Botanik*, xxviii, p. 658, 1895.

[4] Kahlenberg and True, *Botan. Gaz.*, xxii, No. 2, p. 81.

[5] Heald, *ibid.*, xxii, No. 2, p. 125.

of different salts of copper, are equally poisonous for plants. In 1896 also, Paul and Krönig[1] showed that, in general, the more highly ionised a salt is, such as a salt of a heavy metal, the more powerful is its toxic action for bacteria and, therefore, the more intense is its action as a disinfectant. In 1897 Loeb[2] showed that the absorption of water by muscles immersed in solutions of strong inorganic acids could be referred to the hydrion, since equal quantities of hydrion in a given volume of solutions of these acids produced quantitatively equal effects; in the same way the absorption of water in solutions of strong inorganic bases could be referred to the hydroxidion.

Subsequent investigations by Maillard[3] on the toxicity of solutions of copper sulphate to which sulphates of the alkalies had been added; by Eckardt[4] on the relation of the velocity of diffusion to toxicity; by Loeb[5] on the absorption of water by muscles in solutions of electrolytes; by Clark[6] on the toxicity of a variety of substances for moulds; by Kahlenberg,[7] Kastle,[8] by Höber and Kiesow,[9] and by Richards[10] on the taste of dissolved electrolytes, and by Loeb[11] placed beyond doubt the importance of ionic dissociation in the physiological action of dissolved electrolytes.

In 1899 Loeb suggested that the ions entering a tissue combined with or altered chemically some constituent of the tissue.

[1] Paul and Krönig, *Zeitschr. f. physik. Chem.*, xxi, p. 411, 1896; *Zeitschr. f. Hyg.*, xxv, p. 1, 1897.

[2] Loeb, *Arch. f. d. ges. Physiol.*, lxix, p. 1, 1897.

[3] Maillard, *Compt. rend. de la soc. biol.*, 10^e série 5 p. 1210, 1898. *Journ. de physiol. et de path. gen.*, i, pp. 651 and 673.

[4] Eckardt, "Ueber die Diffusion und ihre Beziehung zur Giftwirkung," Inaug. Dissert., Leipzig, Bruno Zeckel 1898, quoted after Maillard *Journ. de physiol. et de path. gen.*, i, p. 658.

[5] Loeb, *Arch. f. d. ges. Physiol.*, lxxviii, p. 457, 1898.

[6] J. F. Clark, *Journ. of Physical Chem.*, iii, p. 263.

[7] Kahlenberg, *Bull. Univ. of Wisconsin*, referred to in *Journ. of Physical Chem.*, iii, p. 66, 1899; *Zeitschr. f. physik. Chem.*, xxix, p. 343, 1899; *Journ. of Physical Chem.*, iv, p. 33, 1900.

[8] Kastle, *Amer. Chem. Journ.*, xx, p. 468, 1898.

[9] Höber and Kiesow, *Zeitschr. f. physik. Chem.*, xxvii, p. 601, 1898.

[10] T. W. Richards, *Amer. Chem. Journ.*, xx, p. 121.

[11] J. Loeb, *Arch. f. d. ges. Physiol.*, lxxv, p. 303, 1899.

He based his argument in the first place upon the dependence of muscular tissue upon the ions diffusing into it,—a dependence which had previously been established by the experiments of Biedermann,[1] Ringer,[2] Howell and Cooke,[3] Locke,[4] and Loeb,[5] and in the second place upon the definite reactions called forth by definite ions.[6] In a later paper he concluded that the ions[7] on entering a tissue combined with some constituent of the tissue, presumably protein, and he conferred upon these compounds the name ion-proteids. Independently and almost simultaneously Pauli[8] arrived at identical conclusions and also gave the name ion-proteids to the compounds formed, while Richards also pointed out, in a paper which appeared at the same time as Loeb's, that some of the phenomena of taste could best be accounted for by supposing that the ions of an electrolyte entered into combination with some constituent of the tissues affected.[9]

In former papers[10] I have endeavored to apply the ion-proteid theory to the problems of excitation and contraction and I have pointed out that the nature of the ion-proteid formed under the influence of an electrolyte is probably determined, in general, by the relative velocities of the ions into which the electrolyte dissociates, since the swifter-moving ions probably diffuse into the tissue in greater numbers than the slower-moving ions, and the ions present in the tissue in the greater mass might be expected, in general, to take the greater share of the proteid; while

[1] Biedermann, *Sitzungsber. d. wien. Akad.*, lxxxii, Part 3, 1880.

[2] Ringer, *Journ. of Physiol.*, iii, pp. 195 and 380; *ibid.*, iv, pp. 29, 222, and 370; *ibid.*, vi, p. 154.

[3] Howell and Cooke, *ibid.*, xiv, p. 189.

[4] Locke, *ibid.*, xviii, p. 332.

[5] Loeb, see papers cited.

[6] J. Loeb, *Arch. f. d. ges. Physiol.*, lxxv, p. 303, 1899; *Festschrift für Professor Fick*, Braunschweig, p. 101, 1899.

[7] J. Loeb, *Amer. Journ. of Physiol.*, iii, p. 327, 1900.

[8] W. Pauli, *Ueber Physikalisch-Chemische Methoden und Probleme in der Medizin*, Wien, 1900.

[9] T. W. Richards, *Journ. of Physical Chem.*, iv, p. 207, 1900.

[10] *Trans. Roy. Soc. of So. Australia*, xxix, p. 1, 1905; *Arch. f. d. ges Physiol.*, cx, p. 610, 1905; this Journal, i, p. 185, 1906; *Biol. Bull.*, xi p. 53, 1906.

the potential difference which would tend to be set up between the tissue and the solution by an excess of ions of one sign would be annulled, as far as those which actually combine are concerned, by the splitting off from the protein of hydrogen or hydroxyl ions of opposite sign.[1]

I have further shown,[2] by means of experiments on the influence of electrolytes upon the staining of tissues by iodine-eosin and by methyl green and on the influence of electrolytes upon the toxicity of alkaloids, that, in general, the acid or basic characteristics of a tissue are largely influenced by the acid or basic properties of the swifter-moving ion in the solution bathing the tissue, presumably because the acid or basic ion diffusing into the tissue in excess confers acid or basic properties respectively upon the constituent of the tissue with which it is combined. Exceptions, as in the case of a tissue bathed in a solution of a salt of a heavy metal, generally admit of other explanations, such as the insolubility of one of the ion-proteids and the consequent irreversibility of the reaction.

Up to this point in the genesis of the ion-proteid theory, however, no assumptions whatever have been made as to the chemical nature of the ion-proteid compounds. It is true that the intimate dependence of the characteristics of a tissue upon the nature of the solution in which it is bathed shows that the ions in the compound are readily substituted, the one for the other, but this throws little light upon their chemical nature.[3]

[1] I find that Tschagowetz, *Journ. Russ. Soc.*, xxviii, 1, p. 657, 1896 (reviewed in the *Journ. of Physical Chem.*, i, p. 317), anticipated me in applying Nernst's formula for the potential difference at the surface of two solutions to the demarcation current in muscle, but he does not appear to have taken into consideration the formation of compounds between the protein and the ion of the other electrolytes present.

[2] This Journal, i, pp. 279 and 507, 1906.

[3] The recent very interesting researches of W. A. Osborne (*Journ. of Physiol.*, xxxiv, p. 84, 1906), strongly support the ion-proteid hypothesis. The interchange of ions between a slightly diffusible protein salt on one side of a membrane and a readily diffusible electrolyte on the other, which Osborne has observed, is precisely what Loeb assumed, when he put forward the ion-proteid hypothesis, to take place in the more complex but analogous case of a cell bathed in a solution of electrolytes, and this has been the main assumption of the ion-proteid theory throughout its development.

(b) *Molecular Compounds of Protein with Electrolytes.*

On the other hand, there have long been described numerous molecular compounds between various protein bodies and other electrolytes. The well-known compounds of proteins with acids and alkalies are examples of this type of compounds. The older literature on the compounds with acids has been collected by Sjöqvist,[1] while in more recent times the work of Sjöqvist,[2] Cohnheim,[3] Cohnheim and Krieger,[4] Erb,[5] Bugarsky and Liebermann,[6] von Rhorer,[7] Ley,[8] Hardy,[9] and Mellanby[10] on albumins and globulins, that of Söldner,[11] van Slyke and Hart,[12] and Long[13] on casein, of Paal,[14] Sjöqvist,[15] Cohnheim,[16] Bugarsky and Liebermann,[17] Cohnheim and Krieger,[18] Erb,[19] von Rhorer,[20] and Sollmann[21] on albumoses and peptones, and that by Erb[22] and by T. B. Osborne[23] on edestin has demonstrated that these protein substances act as bases in the presence of acids and combine with them. On the other hand the work of Bugarsky and

1 Sjöqvist, *Skand. Arch. f. Physiol.*, v, p 277, 1894.
2 Sjöqvist, *loc. cit.*, also *Skand. Arch. f. Physiol.*, vi, p. 255, 1895.
3 O. Cohnheim, *Zeitschr. f. Biol.*, xxiii, p. 489, 1896.
4 O. Cohnheim and Krieger, *ibid.*, xl, p. 95, 1900.
5 Erb, *ibid.*, xli. p. 309, 1901.
6 Bugarsky and Liebermann, *Arch. f. d. ges. Physiol.*, lxxii, p. 51, 1898.
7 von Rhorer, *ibid.*, xc, p. 368, 1902.
8 Ley, *Zeitschr. f. physik. Chem.*, xxx, p. 193, 1899.
9 W. B. Hardy, *Journ. of Physiol.*, xxxiii, p. 251, 1905.
10 J. Mellanby, *ibid.*, xxxiii, p. 338, 1905.
11 Söldner, *Jahresber. d. Anat. u. Physiol.*, xvii, p. 314, 1889.
12 van Slyke and Hart, *Amer. Chem. Journ.*, xxxiii, p. 461, 1905.
13 Long, *Journ. of the Amer. Chem. Soc.*, xxviii, p. 372, 1906.
14 Paal, *Ber. d. deutsch. chem. Gesellsch.*, xxv, 1, p. 1202, 1892; *ibid.*, xxvii, 2, p. 1827, 1894.
15 Sjöqvist, *loc. cit.*
16 Cohnheim, *loc. cit.*
17 Bugarsky and Liebermann, *loc. cit.*
18 Cohnheim and Krieger, *loc. cit.*
19 Erb, *loc. cit.*
20 von Rhorer, *loc. cit.*
21 Sollmann, *Amer. Journ. of Physiol.*, vii, p. 199, 1902.
22 Erb, *loc. cit.*
23 T. B. Osborne, *Zeitschr. f. physiol. Chem.*, xxxiii, pp. 225 and 240, 1901.

Liebermann,[1] Spiro and Pemsel,[2] Hardy,[3] and Mellanby[4] on albumins and globulins, that of Maas,[5] Volhard,[6] and Sollmann[7] on albumoses and peptones, that of Hammarsten,[8] Söldner,[9] Laqueur and Sackur,[10] Sackur,[11] van Slyke and Hart,[12] and others, to which reference will be made in the sequel, upon casein, and that of Biernacki,[13] Bayliss and Starling,[14] and of Vernon[15] upon the retardation of the rate of destruction of trypsin by alkali after the addition of proteins has shown that these protein substances act as acids in the presence of bases and combine with them.

Not only, however, are compounds of proteins with acids and bases known, but also molecular compounds of proteins with salts (Pauli).[16] Doubt was thrown upon the validity of Pauli's views in this matter by the experiments of Bugarsky and Liebermann,[17] Bugarsky and Tangle,[18] Stewart,[19] and Fano and Enriques,[20] who found no depression or, at the most, a very slight

1 Bugarsky and Liebermann, *loc. cit.*

2 Spiro and Pemsel, *Zeitschr. f. physiol. Chem.*, xxvi, p. 233, 1898.

3 Hardy, *loc. cit.*

4 Mellanby, *loc. cit.*

5 Maas, *Zeitschr. f. physiol. Chem.*, xxxix, p. 61, 1900.

6 Volhard, *Münchener med. Wochenschr.*, No. 50, 1903, reviewed in *Biochem. Centralbl.*, ii, p. 231.

7 Sollmann, *loc. cit.*

8 Hammarsten, *Jahresber. f. Thierchem.*, iv, p. 146, 1874.

9 Söldner, *Landwirthsch. Versuchsstation*, 35 separat. 18, quoted after Hammarsten, *Textbook of Physiological Chemistry*, trans. by Mandel, p. 441, 1904.

10 Laqueur and Sackur, *Beitr. z. chem. Physiol. u. Path.*, iii, p. 184, 1902.

11 Sackur, *Zeitschr. f. physikal. Chem.*, xli, p. 679, 1902.

12 van Slyke and Hart, *Amer. Chem. Journ.*, xxxiii, p. 461, 1905.

13 Biernacki, *Zeitschr. f. Biol.*, xxviii, p. 55, 1891.

14 Bayliss and Starling, *Journ. of Physiol.*, xxx, p. 61, 1903.

15 Vernon, *ibid.*, xxxi, p. 346, 1904.

16 W. Pauli, *Arch. f. d. ges. Physiol.*, lxxviii, p. 318, 1899; *Beitr. z. chem. Physiol. u. Path.*, iii, p. 225, 1903; *ibid.*, vii, p. 531, 1906. Huiskamp, *Zeitschr. f. physiol. Chem.*, xxxiv, p. 32, 1901.

17 Bugarsky and Liebermann, *Arch. f. d. ges. Physiol.*, lxxii, p. 51, 1898.

18 Bugarsky and Tangle, *ibid.*, lxxii, p. 540. 1898.

19 Stewart, *Journ. of Physiol.*, xxiv, p. 216, 1899.

20 Fano and Enriques, *Rendiconti della R. Accademia dei Lincei classe di Scienze fisiche*, xii, 2 Sem., 1903, quoted from *Biochem. Centralbl.*, ii, p. 304.

depression of conductivity on adding proteins to solutions of salts. Later, Stewart found a marked rise in conductivity upon the precipitation by heat of muscle-proteids from their solution in water or in magnesium sulphate,[1] but this throws no light upon thé question under consideration, for secondary changes are probably brought about in heat-coagulation with the liberation of ionised substances. The researches of Hardy,[2] and of Mellanby,[3] appear, however, to establish the existence of these neutral salt compounds, while the results of Arny and Pratt[4] appear to establish the existence of such compounds with casein. The results of Bugarsky and Liebermann, to which I have referred, may, as I have pointed out elsewhere,[5] admit of an explanation other than the non-existence of compounds of protein with salts. The compounds of proteins with heavy metals are well-known.[6] Proteins also form salts with dyes[7] and with alkaloids.[8]

Thus we have two distinct arguments: the one, taking its data from the effects of electrolytes upon organisms, concludes that electrolytes or their constituents form some hitherto chemically undefined compound with some constituent of tissues; while the other, taking its data from the properties of solutions containing proteins and electrolytes, concludes that the latter form more or less definite compounds with the proteins which we may for the present designate "molecular compounds."[9]

[1] Stewart, *Journ. of Physiol.*, xxiv, p. 460.

[2] W. B. Hardy, *Journ. of Physiol.*, xxxiii, p. 251, 1905.

[3] Mellanby, *ibid.*, xxxiii, p. 338, 1905.

[4] Arny and Pratt, *Amer. Journ. of Pharmacy*, lxxviii, p. 121, 1906.

[5] Robertson, *Journ. of Physical Chem*, x, p. 524, 1906.

[6] For literature consult Gustav Mann, *Chemistry of the Proteids*, p. 303, 1906, etc.

[7] For literature consult Gustav Mann, *Chemistry of the Proteids*, p. 327, 1906; *Physiological Histology*, Oxford, p. 211, 1902, etc.

[8] Spiro, *Zeitschr. f. physiol. Chem.*, xxx, p. 182, 1900. W. A. Osborne *Journ. of. Physiol.*, xxvii, p. 398, 1901. For further literature consult Robertson, this Journal, i, p. 507, 1906.

[9] This term, as I will show more fully later on, is only applicable to one of the compounds between electrolytes and the type of proteins which I have elsewhere designated Class I.

In a recent paper[1] I have endeavored to establish a connection between these two arguments, and I have suggested that the effects of electrolytes upon tissues may possibly be attributed to alterations in the equilibrium of the proteins regarded as amphoteric electrolytes (type HXOH), that the ion-proteids are, in reality, salts of the ampholates, that is, of the proteins dissociating as acids or as bases ("ampho-salts," types: $Na^{+}+XOH'$ $HX^{+}+Cl'$), and that the "non-dissociable" ion-proteid compounds[2] which exist in tissues are, in reality, ampho-salts dissociating as acids or as bases (types: $NaX^{+}+OH'$, $H^{+}+XCl'$).

(c) *Objects of this Investigation.*

The present investigation was undertaken with the object, in the first place, of ascertaining the relation between the ion-proteids and the molecular compounds of proteins with electrolytes, and, in the second place, of ascertaining, under more exactly defined conditions than hitherto, the influence of electrolytes upon tryptic digestion.

Casein was chosen as affording the most favorable point of attack; in the first place, because its compounds with acids and alkalies have already been the subject of very numerous investigations, so that we have a comparatively large number of data at our disposal, and, in the second place, because, as we shall see, casein lends itself to accurate quantitative estimation.

II. SOME CHEMICAL PROPERTIES OF CASEIN.

(a) *Nomenclature.*

In studying the chemical properties of casein we are confronted at the outset with no little confusion in nomenclature. The term casein was restricted by Hammarsten to the substance normally present in milk, while the coagulum formed when this substance is acted on by rennet was termed by him *Käse*.[3] In 1884 it was proposed by E. Schultz and Röse to call Hammars-

[1] Robertson, *Journ. of Physical Chem.*, x, p. 524, 1906.

[2] J. Loeb, *Dynamics of Living Matter*, p. 72, 1906.

[3] Hammarsten, *Jahresber. f. Thierchem.*, iv, p. 138, 1874. Sebelien, *Journ. of Physiol.*, xii, p. 95, 1891.

ten's Käse, *paracasein*,[1] and this suggestion has been widely accepted; according to Mann,[2] however, Hammarsten must have antedated Schultz and Röse in utilizing the terms casein and paracasein. In 1888, Foster,[3] without referring to the nomenclature adopted by Schultz and Röse, proposed to call the product of the action of rennet upon "the natural soluble casein" "*tyrein*," while in 1890 Halliburton, stating that Foster, so far as he had been able to find, was the only previous author who had recognized the distinction between the casein normally present in milk and the curd produced by rennet, proposed to call the substance normally present in milk *caseinogen*, restricting the term *casein* to the curd produced by rennet.[4] This nomenclature of Halliburton's is analogous to that applied to other substances, such as fibrinogen and fibrin, myosinogen and myosin, and which has also been applied to various zymogens and enzymes such as trypsinogen and trypsin, in each case the suffix, *-ogen*, denoting a precursor. Other nomenclatures have at various times been suggested but none of them appears to have claimed many adherents.[5]

While the nomenclature suggested by Halliburton possesses some advantages it has not been universally received, and since that proposed by Schultz and Röse appears to have been more generally adopted and, moreover, possesses the right of priority, I have adopted it here in the modified form suggested by van Slyke and Hart.[6]

We shall therefore restrict the term "*casein*" to the free proteid, unaltered by rennet. The compound with calcium hydrate which is neutral to litmus, and which exists in cows milk, will be called "*calcium casein*," "*neutral calcium casein*," or "*neutral calcium caseinate*," the compound with calcium hydrate which is neutral to phenolphthalein will be called "*basic*

[1] E. Schultz and Röse, *Landwirtsch. Versuchsstationen*, xxxi, p. 131, 1884–1885, quoted after Sebelien, *loc. cit.*

[2] Gustav Mann, *Chemistry of the Proteids*, p. 491, 1906.

[3] Foster, *Textbook of Physiology*, 5 Ed., ii, p. 376, 1888.

[4] Halliburton, *Journ. of Physiol.*, xi, p. 448, 1890.

[5] Consult Raudnitz, *Ergebnisse der Physiologie*, ii, 1, p. 193, 1903.

[6] Van Slyke and Hart, *Amer. Chem. Journ.*, xxxiii, p. 461, 1905.

calcium casein," or "*basic calcium caseinate*," while the compounds of casein with acids will be termed "*casein hydrochloride*," "*casein sulphate*," etc. The product of the action of rennet upon calcium casein we shall only refer to incidentally, but the free proteid will be termed "*paracasein*," and the same nomenclature as that applied to the compounds of casein will be used to designate the compounds of paracasein; thus the compound with calcium hydrate which is neutral to litmus will be termed "*calcium paracasein*," "*neutral calcium paracasein*," or "*neutral calcium paracaseinate*," etc.

(b) *General Properties.*

Casein, in the dry state, forms a white powder which is insoluble in water[1] and is hygroscopic. I find that purified casein dried for about five hours at 70° to 80° C. loses 5.8 per cent. of its weight. I could not detect any appreciable further loss of weight on continuing the drying at 70° C., but Béchamp found that at 140° C. casein lost 16.5 per cent. of its weight in the form of water.[2] According to the majority of observers, casein reddens litmus paper. W. A. Osborne, however, denies this. The reasons for this difference of opinion lie, I think, in the following facts:—A suspension of casein, thoroughly shaken up in distilled water, reddens litmus paper wherever the suspended particles touch it, but if this suspension be filtered the filtrate contains no detectable casein and is neutral to litmus. The casein is possibly soluble to a very slight extent in distilled water but this slight amount is practically entirely concentrated at the surface of the water immediately in contact with the solid particles owing to the diminution in surface energy which is thus brought about,[3] so that the litmus is only reddened where it touches these surfaces. Gibbs has pointed out that such an interfacial transition-layer may be regarded as a distinct phase

[1] Laqueur and Sackur, according to Hammarsten, *Textbook of Physiological Chemistry*, trans. by Mandel, p. 441, 1904. Cohnheim, *Chemie der Eiweisskörper*, Braunschweig, p. 173, 1900. W. A. Osborne, *Journ. of Physiol.*, xxvii, p. 398, 1901.

[2] Béchamp, *Bull. soc. chim.*, (3), xi, p. 152, 1894.

[3] See Whetham, *A Treatise on the Theory of Solutions*, p. 98, 1902.

having its own characteristic equation.[1] It may be pointed out here that, in regard to the properties which we have so far dealt with, casein resembles the other phospho-globulins,[2] to which class it belongs, and also, possibly to a less extent, globulin.[3] The decomposition-products of casein are fully discussed in Gustav Mann's *Chemistry of the Proteids*.[4]

The base-free casein is soluble in warm 5 per cent. salt solution and in hot 50 per cent. alcohol; when freshly prepared and warmed it is very plastic and is capable of being drawn out into long, fine threads.[5]

Casein acts as an acid in expelling carbon dioxide from carbonates and bicarbonates, forming a salt with the base,[6] and in this way, according to Osborne,[7] the ammonium, potassium sodium, lithium, magnesium, strontium, and calcium caseinates can be prepared, while by acting upon the casein with barium hydrate barium caseinate can be prepared. Osborne also states that casein forms salts with alkaloids such as caffein and strychnin.[8] The solutions of the neutral caseinates of the alkaline earths form a haptogen film on the surface on heating (Osborne) and are converted by rennet into paracasein compounds,[9] but a coagulum is only formed by the action of rennet upon a solution of calcium casein in the presence of excess of soluble calcium salts[10]; solutions of the caseinates of the alkalies do not form coagula when acted on by rennet.[11] The solutions

[1] J. Willard Gibbs, *Trans. Connect. Acad.*, iii, p. 380, 1877.

[2] Compare Gustav Mann, *Chemistry of the Proteids*, p. 394, 1906.

[3] W. B. Hardy, *Journ. of Physiol.*, xxxiii, p. 268, 1905.

[4] Page 70.

[5] van Slyke and Hart, *Amer. Chem. Journ.*, xxxiii, p. 472, 1905.

[6] Söldner, quoted after van Slyke and Hart, *loc. cit.*, p. 465.

[7] W. A. Osborne, *loc. cit.*

[8] Wroblewski, *Zeitschr. f. physiol. Chem.*, xxi, p. 1, 1895, had previously stated that nicotin combines with casein.

[9] Ringer, *Journ. of Physiol.*, xi, p. 464, 1890; and xii, p. 164, 1891. W A. Osborne, *ibid*, xxvii., p. 398, 1901. van Slyke and Hart, *Amer. Chem. Journ.*, xxxiii, p. 484, 1905.

[10] Ringer, *Journ. of Physiol.*, xi, p. 464, 1890. van Slyke and Hart, *loc. cit.*

[11] W. A. Osborne, *loc. cit.* Ringer, *Journ. of Physiol.*, xii, p. 164, 1891, alludes to the difficulty of precipitating paracasein by calcium chloride when it is dissolved in sodium bicarbonate or hydrate.

of the caseinates of the alkaline earths and alkaloids are markedly opalescent, and these caseinates are precipitated from their solution by the addition of any finely divided insoluble substance or by passage through a clay filter, while on warming their solutions to 35°–45° C. a marked turbidity occurs which disappears on cooling, a phenomenon which Osborne attributes to hydrolysis on heating, setting free the insoluble casein.

The salts of the alkalies and ammonium give clear solutions, are not precipitated by finely divided, inscluble substances or by passing through a clay filter, and do not (except in the case of lithium caseinate) show any increase in turbidity on warming.[1]

(c) *Quantitative Estimation of Casein.*

The methods hitherto used for the estimation of the amount of casein destroyed in digests have chiefly depended upon alterations in some physical properties of the solutions, such as viscosity[2] or electrical conductivity,[3] in which the chemical meaning of the quantity measured is not very clear, while Plimmer and Bayliss[4] have utilized the splitting off of "soluble phosphorus" as a means of estimating the rate at which casein is digested.

Volhard,[5] however, used casein hydrochloride in his digestion experiments, precipitated the undigested casein by means of sodium sulphate, and titrated, using the amount of free acid in the filtrate as an estimate of the amount digested. This method has been criticised by Glässner.[6]

None of the methods described above appear to admit of a very high degree of accuracy so far as the estimation of the amount of casein actually digested is concerned. Gravimetric methods

[1] W. A. Osborne, *loc. cit.*

[2] Emil Fischer and Abderhalden, *Zeitschr. f. physiol. Chem.*, xl, p. 215, 1903.

[3] Bayliss, *Arch. des sci. biol.*, xi, Suppl., p. 261, 1904. V. Henri and des Bancels, *Compt. rend. d. soc. biol.*, lv, p. 789, 1903.

[4] Plimmer and Bayliss, *Journ. of Physiol.*, xxxiii, p. 439.

[5] Volhard, *Münch. med. Wochenschr.*, Nos. 49 and 50, 1903, quoted from *Biochem. Centralbl.*, ii, p. 231.

[6] Glässner, *Münch. med. Wochenschr*, lii, 1903.

have been employed to estimate casein, for instance by W. A. Osborne,[1] but they involve a large expenditure of time and also some uncertainties. The researches of van Slyke and Hart,[2] however, appear to indicate the possibility of estimating casein volumetrically with a greater approach to accuracy than hitherto.

These observers have confirmed Söldner's[3] observation that casein combines with about 2.4 per cent. of calcium oxide forming a compound neutral to phenolphthalein (basic calcium caseinate). In other words, one gram of casein neutralizes to phenolphthalein an amount of calcium hydrate equivalent to 8 c.c. of $\frac{N}{10}$ hydrochloric acid. This also corresponds exactly with the results obtained by Laqueur and Sackur.[4]

The method actually employed in the majority of the experiments was as follows:

The casein in 200 c.c. of the solution under investigation was precipitated by the addition of about 15 c.c. of $\frac{N}{10}$ acetic acid (made up approximately by diluting 10 c.c. of Kahlbaum's glacial acetic acid to 1750 c.c.) In the earlier experiments on the digestion of casein by trypsin saturated solutions of casein in $\frac{N}{100}$ alkali were used and it was found experimentally that 15 c.c. of the acetic acid solution was the least amount which gave a quick and complete precipitation; in the later experiments more dilute solutions of casein were employed, but as it was found that excess of acetic acid did not introduce any appreciable error into the estimation, the same proportion of $\frac{N}{10}$ acetic acid solution to the casein solution was adhered to throughout, except in a few experiments in which solutions of casein in alkali stronger than $\frac{N}{100}$ were under investigation. The precipitate produced by the acetic acid was collected on a filter and washed in a stream of distilled water for an hour or for a longer period if the precipitate was bulky; the distilled water was siphoned off from a large vessel and its outflow into the filter regulated by a screw-clamp attached to a rubber tube until the rates of entry into and of exit from the filter were equal. The filter containing the precipitate was then placed in a beaker and a given amount (as a rule, 25 c.c.) of calcium hydrate solution (approximately $\frac{N}{25}$) which had been standardized against standard $\frac{N}{10}$ hydrochloric acid was run in and distilled water added till the volume in the beaker was about 200 c.c. The filter paper was then macerated in the solution by stirring with a glass rod the end of which was protected

[1] W. A. Osborne, *Journ. of Physiol.*, xxvii, p. 398, 1901.
[2] van Slyke and Hart, *Amer. Chem. Journ.*, xxxiii, p. 461, 1905.
[3] Söldner, *Landw. Versuchsstat.*, xxxv, p. 351, 1888.
[4] Laqueur and Sackur, *Beitr. z. chem. Physiol. u. Path.*, iii, p. 196, 1903.

with rubber tubing, which served the double purpose of guarding against cracking the beaker and of enabling one to rub off the sides of the beaker any particles of casein adhering to them. This maceration was continued until a fairly uniform pulp suspended in an opalescent solution was obtained and no particles of undissolved casein were left; since the particles of casein quickly fall to the bottom of the beaker and are different in color from the particles of filter paper the point of complete solution is readily determined and usually occurs some time before the filter paper is thoroughly macerated into a pulp. The filter papers used throughout these experiments were Schleicher and Schüll's No. 589 "black band" papers as these retained the precipitate well, filtered rapidly, and readily underwent maceration. Since these filter papers are practically ash-free their influence upon the reaction of the solution is negligible. To this solution ten drops of an alcoholic solution of phenolphthalein (approximately one per cent. The same solution and the same dropper were used throughout. The phenolphthalein was de Haen's.) were now added and the standard $\frac{N}{40}$ hydrochloric acid was run in until the solution was rendered just colorless, so that one drop of $\frac{N}{25}$ calcium hydrate solution brought back a just perceptible color; this was taken as the neutral point. It is necessary thus to describe in detail the exact procedure as the change in color of the solution is gradual and extends over the addition of from 0.5 to 1 c.c. of $\frac{N}{40}$ hydrochloric acid. Some point has, therefore, more or less arbitrarily to be decided on and the above procedure, under the conditions of volume, etc., outlined above, gave, as we shall see, results in accordance with those of van Slyke and Hart and of Laqueur and Sackur to which I have alluded.

Knowing the number of cubic centimeters of the standard acid required to neutralize 25 c.c. of the standard lime-water solution, the difference between this number and that required to neutralize 25 c.c. of the calcium hydrate solution plus the casein, multiplied by a factor, gives the number of grams of casein in the original 200 c.c. of solution. The exact factor depends, of course, upon the concentration of the acid employed, but supposing it to be accurately $\frac{N}{40}$, since 0.125 gram of casein is equivalent to 1 c.c. of $\frac{N}{10}$ acid, according to the measurements of van Slyke and Hart and of Laqueur and Sackur, therefore 1 c.c. of $\frac{N}{40}$ hydrochloric acid is equivalent to the amount of calcium hydrate neutralized by 0.03125 gram of casein; multiplying, therefore, the difference between the number of cubic centimeters of acid required to neutralize 25 c.c. of the calcium hydrate solution and that required to neutralize 25 c.c. of the same solution after the casein has been dissolved in it by 0.03125 gives us the number of grams of casein in the original solution.

The hydrochloric acid solution was originally standardized in two ways. In the first place a decinormal sodium hydrate solution was made up and titrated with phenolphthalein indicator against a standard decinormal hydrochloric acid solution which had been used in this laboratory for standardizing solutions and was considered to be exact. The $\frac{N}{40}$ hydro-

chloric acid was now standardized against this alkali, using phenolphthalein as indicator. In the second place, a solution of $\frac{N}{10}$ silver nitrate was made up and standardized with chromate indicator against carefully dried and weighed Kahlbaum's sodium chloride. A known volume of the $\frac{N}{10}$ hydrochloric acid solution was then neutralized by Kahlbaum's sodium bicarbonate and titrated against this silver nitrate with chromate as indicator. The two results agreed almost exactly and their mean was taken as correct. The calcium hydrate solutions were made up by igniting Kahlbaum's calcium oxide and nearly saturating water with the calcium hydrate formed on adding to it the ignited calcium oxide; they varied in concentration between $\frac{N}{23}$ and $\frac{N}{25}$. They were then standardized after filtration, against the $\frac{N}{10}$ hydrochloric acid: subsequent solutions of hydrochloric acid were standardized against the calcium hydrate solution and *vice versa*, while at intervals the hydrochloric acid solutions were rechecked against the silver nitrate, which, in its turn, was several times restandardized against Kahlbaum's sodium chloride.

Before quoting actual results several sources of error in this method have to be considered.

In the first place, unless certain precautions are taken, the precipitation by acetic acid may not be complete, while it is obvious that the whole accuracy of the method depends upon the completeness of this precipitation. If 15 c.c. of $\frac{N}{10}$ acetic acid be added to 200 c.c. of a saturated solution of casein in lime-water, it may happen that no precipitation occurs at all, although the solution increases markedly in opalescence and the amount of acetic acid present may be greatly in excess of that required to precipitate all the casein. Indeed if such a "solution" be left undisturbed and has not previously undergone digestion, and no other substances be present (such as neutral salts), hardly any precipitate may be deposited for at least twenty-four hours. The change from the finely-suspended condition to the precipitated condition is therefore one into which a time factor enters. Even vigorous stirring of such a "solution" may not result in a complete precipitation, the supernatant fluid remaining opalescent. If, however, the acetic acid be added slowly and the solution vigorously stirred or shaken just while the increase in opalescence is taking place, minute granules begin to appear, and on then adding the excess of acetic acid a flocculent precipitate is at once obtained and the supernatant fluid is left quite clear. This phenomenon may possibly be a particular case of the general law enunciated by Hardy for colloidal solutions, namely, that the more slowly the division into two phases occurs the smaller and less curved is the surface of separation.[1] Freundlich has also drawn attention to similar phenomena.[2]

Small quantities of neutral salts, as also the products of digestion, appear to accelerate the change from the finely-suspended condition to

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[1] W. B. Hardy, *Journ. of Physical Chem.*, iv, p. 258, 1900.
[2] Freundlich, *Zeitschr. f. physik. Chem.*, xliv, p. 129, 1903.

the precipitated condition; at all events such difficulties were seldom met with in solutions which had been digested or to which salts (concentration $\frac{N}{40}$ and upwards) had been added. On quickly adding excess of acetic acid, without shaking, the precipitate might not come down at once, it is true, but on standing or on slight shaking it at once came down. In any case the filtrate is more likely to be perfectly clear if the solution be left standing for about an hour after adding the acetic acid. In all cases if the filtrate, on comparing with distilled water, was found to be at all opalescent it was refiltered, which usually succeeded, or, if this failed, the experiment was rejected.

Another source of error is the tendency, which the casein precipitate occasionally displays, to pass through the filter after the original filtrate has come through clear and while the precipitate is being washed. This rarely occurred and when it did refiltration of the washings and rewashing usually proved sufficient to secure the retention of the precipitate. I was unable to assign a reason for this when it did occur.

Another possible source of error is the solvent action on casein of excess of acetic acid which is claimed by some investigators. As I have pointed out, however, I have been unable to detect this solvent action of acetic acid. Moreover, duplicate determinations were occasionally made in which different quantities of acetic acid had been used for precipitation and the results of such determinations did not differ in any definite sense nor by more than the experimental error.

Another thing which has to be guarded against is the possibility of incomplete extraction of the acetic acid from the precipitate by the washing. If less than 200 milligrams of casein are collected on a 15 cm. filter one hour of washing is usually ample, unless an unusual excess of acid is employed. For quantities of casein greater than this an hour and a half or two hours' washing must be employed.

The error due to the absorption of acid or base by the filter paper must be negligible, since, according to Evans' results, one gram of filter paper would only absorb half a milligram of hydrochloric acid from a fortieth-normal solution.[1]

As no precautions were taken to exclude it, the carbon dioxide in the distilled water employed in these estimations provided another possible source of error. The error was approximately constant, however, as the volume of the fluid titrated was kept constant. Moreover, as the standardization of the calcium hydrate solutions was also made in each case with about the same total volume (200 c.c.) of fluid, the error due to carbon dioxide was practically eliminated, for although the actual concentration of the calcium hydrate, as estimated by titration, might be affected, yet the difference between the number of cubic centimeters of standard acid required to neutralize a given amount of lime-water and

[1] Evans, *Journ. of Physical Chem.*, x, p. 290, 1906.

the number required to neutralize the same amount of lime-water, under the same conditions of volume, etc., after the addition of casein, would not be subject to any appreciable error. The titrations, however, as is usual when phenolphthalein is used as indicator, could not be carried on successfully in a room containing several lighted bunsen burners.

The following table will serve to illustrate the results which are obtained by this method. Since it was important for the purposes of these investigations to ascertain whether the nature of the solvent had any influence upon the precipitation by acetic acid, or otherwise affected the accuracy of the method, varying solvents were used from time to time.

The figures in the first column are the weighed amounts, in grams, minus 5.8 per cent., which, as we have seen, is the amount of water driven off by drying at 70°C.

TABLE I.

Weighed Amount of Casein in 100 c.c.	Solvent.	Estimated Amount of Casein 100 c.c.
6.9	$\frac{N}{25}$ $Ca(OH)_2$	6.77
1.63	$\frac{N}{100}$ $Ca(OH)_2$	1.60
0.802	$\frac{N}{200}$ $Ca(OH)_2$	0.788
0.364	$\frac{N}{1250}$ $Ca(OH)_2$	0.360
0.342	$\frac{N}{1250}$ $Ca(OH)_2$	0.340
0.15	$\frac{N}{50}$ $Ca(OH)_2$	0.155
0.113	$\frac{N}{1250}$ $Ca(OH)_2$ + $\frac{N}{10}$ NH_4NO_3	0.111
0.113	$\frac{N}{1250}$ $Ca(OH)_2$ + $\frac{N}{10}$ CH_3COONa	0.111
0.094	$\frac{N}{30}$ $Ca(OH)_2$ + $\frac{N}{12}$ HCl	0.097 [1]

[1] It is necessary to nearly neutralize an acid solution of casein before attempting to precipitate with acetic acid.

No indication of a tendency on the part of added electrolytes to inhibit the precipitation of the casein by acetic acid was observed except in the cases of ferric and cupric chlorides. Potassium cyanide renders precipitation difficult, but approximately quantitative results were obtained in estimating the casein in potassium cyanide solutions, although the large excess of acetic acid which it was necessary to use for precipitation rendered its complete extraction from the precipitate by washing somewhat difficult.

The casein used throughout these experiments was the casein prepared by Hammarsten's method, sold by Messrs. Eimer & Amend. This preparation contained a small percentage of a water-soluble acid, presumably acetic acid. After triturating 100 grams with several liters of water, however, and then washing with alcohol and ether and drying at 40° C., the product no longer gave an acid reaction to distilled water after filtration. This "purified casein" was used for all the experiments on solubility, etc., and for most of the experiments on tryptic digestion. In most of the experiments on the influence of salts upon the velocity of digestion, however, the unpurified substance was used. The minute amount of acetanion thus introduced, however, probably did not appreciably affect the results, for, as we shall see later, a number of experiments on the velocity of autohydrolysis, made both with the purified and the unpurified casein, gave practically identical velocity constants for both preparations.

Some time after I had begun this investigation, a paper by Arny and Pratt appeared in which they describe another method of estimating casein volumetrically.[1] These observers find that casein combines with alums in molecular proportions and that the compound with ferric alum is insoluble; hence, in order to estimate casein, they run into the solution an excess of a solution of ferric alum, filter off the precipitate, and determine the iron left in the filtrate. Judging from their results, this method is very accurate. I did not adopt it, however, in the remainder of the investigation, firstly, because it appeared advisable that all the results should be obtained by a uniform method, and, secondly, because, no factor connecting the amount of casein precipitated with the weight of ferric alum combined having yet been worked out, only comparative and not absolute determinations of casein could have been made. My method is also, possibly, the quicker one of the two.

(*d*) *Solubility in Alkalies.*

As we have stated, casein acts as an acid in the presence of bases, forms salts with the alkalies and alkaline earths, and is able to expel carbon dioxide from their carbonates. The amount of calcium oxide with which casein combines has been the subject of many investigations; Söldner found that 100

[1] Arny and Pratt, *Amer. Journ. of Pharmacy*, lxxviii, p. 121, 1906.

grams of casein combined with an amount of calcium hydrate equivalent to 2.32 grams of calcium oxide to form a compound neutral to phenolphthalein[1]; Courant found that the compound neutral to phenolphthalein contained 2.91 grams of calcium oxide,[2] while the figures given by other observers are: de Jager,[3] 2.4, Timpe,[4] 2.618, Kobrak,[5] 2.98, Laqueur and Sackur,[6] 2.46, van Slyke and Hart,[7] 2.4.

The compound of casein with 2.46 per cent. of calcium oxide or 2.73 per cent. of sodium oxide, neutral to phenolphthalein, is termed by Söldner the basic caseinate, by Courant the tricaseinate of the base, while the compound of casein with 1.55 per cent. of calcium oxide or 1.71 per cent. of sodium oxide, neutral to litmus, is described by Söldner as the neutral caseinate, by Courant as the dicaseinate of the base; according to the determinations of van Slyke and Hart, this compound contains 1.5 per cent. of calcium oxide. In addition to these two classes of compounds a third, acid to litmus and containing 0.961 per cent. of sodium oxide, has been described by Timpe,[8] while, more recently, Long[9] has described "acid salts" of casein, containing twice as much casein as the compound neutral to phenolphthalein; as we shall see, however, the existence of these latter compounds is questionable.

The ammonium caseinate, neutral to litmus (eucasein), contains, according to Béchamp,[10] 1.17 to 1.21 per cent. of ammonia (= 1.94 to 1.99 per cent. of calcium oxide), while Salkowski[11] states that this compound contains only 0.35 per cent. of ammonia

[1] Söldner, *Landw. Versuchsstat.*, xxxv, p. 351, 1888; quoted after van Slyke and Hart, *loc. cit.*

[2] Courant, *Arch. f. d. ges. Physiol.*, l, p. 109, 1891.

[3] L. de Jager, *Nederl. Tijdschr. v. Geneesk.*, ii, p. 253, 1897, quoted from *Jahresber. f. Thierchem.*, xxvii, p. 276, 1897.

[4] H. Timpe, *Arch. f. Hyg.*, xviii, p. 1, 1893, quoted after Raudnitz, *Ergeb. d. Physiol.*, ii, 1, p. 193, 1903.

[5] E. Kobrak, *Arch. f. d. ges. Physiol.*, lxxx, p. 69, 1900.

[6] Laqueur and Sackur, *Beitr. z. chem. Physiol. u. Path.*, iii, p. 193, 1902.

[7] van Slyke and Hart, *loc. cit.*

[8] Timpe, *loc. cit.*

[9] J. H. Long, *Journ. of the Amer. Chem. Soc.*, xxviii, p. 372, 1906.

[10] Béchamp, *Bull. soc. chim.* (3), xi, p. 152, 1894.

[11] E. Salkowski, *Zeitschr. f. Biol.*, xxxvii, p. 415, 1899.

I find that if a given concentration of alkali is "saturated" with casein, that is, if it is shaken up with casein for some time and then filtered through S and S, 589, "black band" papers, the filtrate is always neutral to litmus or to cyanin. This is true for all the concentrations of calcium hydroxide which I have tried, varying between 0.0385 N and about 0.0005 N. The reactions of such solutions to different indicators are as follows:

Phenolphthalein	Colorless.
Tropæolin 000	Orange.
Neutral red	Red.
Cyanin	Very pale blue.
Congo red	Scarlet.

corresponding to a hydrion concentration of 10^{-6} N to 10^{-7} N[1] and to the formation of the neutral calcium caseinate. All attempts to obtain a solution of calcium caseinate of higher acidity after filtration, by means of prolonged shaking, etc., failed; a solution of calcium hydrate, shaken rapidly for over an hour, with excess of casein by means of an electromotor, still gave a filtrate with the same reaction, practically that of conductivity water, nor did the presence of impurities, such as acetic acid, affect the final result as regards hydrion concentration, at all events so far as could be judged by means of the indicators used. Long, in the paper to which I have referred, concluded that he had obtained "acid salts" of casein containing twice as much casein as the basic caseinates, whereas the proportion of the casein in the neutral caseinates to that in the basic caseinates is as three is to two. The evidence upon which he based his conclusion was that he was able "to just dissolve the casein in exactly half the alkali required for the phenolphthalein neutralization", of course the validity of this conclusion depends upon our definition of "solution" which is necessarily arbitrary in such a case. It is true that a compound containing a greater percentage of casein than the neutral caseinate might conceivably exist in the suspension and be separated out by the filtration, but it seems unlikely, especially as the filters used are rapid-filtering. The hydrion concentration of the suspension before

[1] H. Friedenthal, *Zeitschr. f. Electrochem.*, x, p. 113, 1904; B. Fels, *ibid.*, p. 208; E. Salm, *ibid.*, p. 341.

filtration, even after allowing to settle, cannot be trusted to give any reliable information owing to the acid reaction of the suspended particles of uncombined casein.

If "saturation" of a solution of calcium hydrate with casein indicates the formation of the neutral caseinate, the amount of casein dissolved in a given volume should be proportional to the concentration of the calcium hydrate. Now a given volume of such a solution of calcium caseinate requires the addition of a certain number of cubic centimeters of calcium hydrate solution to render it neutral to phenolphthalein, that is, the total number of cubic centimeters of calcium hydrate solution neutralized to phenolphthalein by the casein is the given volume of the caseinate solution plus the number of cubic centimeters of calcium hydrate solution which it is necessary to add to reach neutrality to phenolphthalein. Knowing the number of cubic centimeters of the calcium hydrate solution which is neutralized to phenolphthalein by one gram of casein, we can thus estimate the percentage of casein in the solution. At the same time we can estimate the percentage of casein theoretically on the assumption that the "saturated" solution of casein in the given concentration of calcium hydrate contains casein only in the form of neutral calcium caseinate, that is, the compound containing 1.57 per cent. of calcium oxide.[1]

In the following table the two estimates are compared; the number of grams of casein in the first column was shaken up in 100 c.c. of a calcium hydrate solution of the concentration given in the second column, the solution was then filtered through S and S, 589, "black band" papers and titrated.

TABLE II.

Grams of Casein	Concentration of $Ca(OH)_2$	Grams of Casein Dissolved per 100 c c	
		By Titration.	Theoretical.
15.0	0.0387N.	6.77	6.9
7.5	0.0188N.	3.08	3.35
3.75	4.0091N.	1.604	1.63
1.875	0.0045N.	0.788	0.802

[1] van Slyke and Hart, *Amer. Chem. Journ.*, xxxiii, p. 470, 1905.

We are, therefore, I think, justified in concluding that *the maximum amount of casein which a given concentration of calcium hydroxide will keep in solution is that with which it will combine to form neutral calcium caseinate.*

(*e*) *Solubility in Salts.*

One gram of purified casein was shaken up in 5 c.c. of a normal solution of the salt plus 45 c.c. of distilled water. When the 5 c.c. of normal salt solution was poured on to the casein and allowed to stand for about an hour, if the salt was one of those in which the casein is most soluble, the casein swelled up into a gelatinous mass and absorbed most of the fluid (acetates, propionates, and butyrates of the alkalies, potassium cyanide, etc.). In others (such as chlorides, nitrates, etc.) the casein remained in the form of discrete particles with supernatant fluid. Those solutions in which the most casein was dissolved also foamed most on shaking.

These solutions, after standing an hour or two, were filtered through S and S, 589, "black band" papers, the casein from a known volume precipitated by $\frac{N}{10}$ acetic acid and estimated in the usual way. The following are the results:

TABLE III.

Salt.	Grams of Casein Dissolved in 100 c.c.
$\frac{N}{10}$ $NaCl$	0.[1]
" Na_2SO_4	0.
" $NaNO_3$	0.
" KCl	0.
" $LiNO_3$	0.
" KBr	0.188
" $(COONa)_2$	0.406
" NH_4NO_3	0.451
" CH_3COONa	0.476
" NH_4CNS	0.927
" CH_3CH_2COONa	1.128
" $CH_3CH_2CH_2COONa$	2. or over.[2]
" C_4H_9COONa	2. or over.
" CH_3COOK	2. or over.
" KCN	2. or over.

1 The solubility in the first five solutions in the table is stated to be zero because there was no perceptible opalescence in the filtrate and no precipitate, or at most a slight opalescence, was produced by acetic acid.

2 The solubility in the last four solutions in the table is stated to be 2 per cent. or over because all the casein was dissolved or only a slight slime was left which could not be satisfactorily separated by filtration

The solution in potassium cyanide was of a different character from the others, being yellower and more gummy and viscid.

(f) *Solubility in Alkali Plus Salts.*

One gram of purified casein was shaken up with 25 c.c. of approximately $\frac{N}{250}$ calcium hydrate plus 5 c.c. of a normal solution of the salt, and the volume made up to 50 c.c. with distilled water. After standing for an hour or two the solutions were filtered through S and S, 589, "black band" filters and the casein from a known volume of the filtrate precipitated by $\frac{N}{10}$ acetic acid and estimated in the usual way. The following are the results:

TABLE IV.

Salt.	Grams of Casein Dissolved in 100 c.c.
$\frac{N}{10}$ $CaCl_2$	trace.
No salt	0.238
$\frac{N}{10}$ HCOONa	0.316
" Na_2SO_4	0.361
" NaCl	0.376
" KBr	0.421
" $LiNO_3$	0.436
" $NaNO_3$	0.451
" CH_3COONa	0.541
" NaF	0.556
" NH_4NO_3	0.556
" $KClO_3$	0.677
" $(COONa)_2$	0.782
" Sodium propionate	2. or more
" Sodium butyrate	2. " "
" Potassium acetate	2. " "
" Sodium valerianate	2. " "
" KCN	2. " "

Clearly *the solvent powers of calcium hydrate and the various salts, for casein, are not additive*, that is, the amount of casein dissolved by the two acting together is not the sum of the amounts

dissolved by each acting separately, as it is in the case of globulin for pairs of salts.[1]

A liter of approximately $\frac{N}{250}$ calcium hydrate solution was "saturated" with casein, so that a solution of neutral calcium casein was obtained, and to 25 c.c. of this were added 5 c.c. of normal solutions of various salts, and the volume made up to 50 c.c. with distilled water. In this way it could be seen that *in equally concentrated solutions of casein the absence of opalescence runs parallel with the solubility of the casein in the various solutions.* Thus the solutions containing no salt, potassium chloride, and sodium fluoride were the most opalescent, in the order named, while the solutions containing potassium acetate, sodium butyrate, and potassium cyanide were almost clear.

On adding calcium chloride to a solution of neutral calcium caseinate the casein is precipitated, as has been noticed by other observers. The addition of $\frac{M}{50}$ calcium chloride is sufficient to precipitate practically all the casein from 50 c.c. of a $\frac{N}{400}$ calcium hydrate solution "saturated" with casein, although even at a concentration of $\frac{2N}{5}$ calcium chloride, after filtering, the filtrate still gives a slight opalescence with acetic acid. Presumably this precipitate is due to the "salting out" of the calcium caseinate, using the term "salting out" as synonymous with precipitation due to a common ion. The casein was not precipitated by any of the other salts in the concentrations tried.

(g) *Reactions of Solutions of Casein to Indicators.*

One liter of $\frac{N}{20}$ sodium hydrate was "saturated" with casein and filtered through glass wool; to this, one liter of $\frac{N}{20}$ sodium hydroxide was added and the resulting alkaline solution filtered through paper. To 25 c.c. of this solution varying amounts of $\frac{N}{10}$ hydrochloric acid were added and water added to bring up the total volume to 50 c.c. The proportions of indicator to solution used were the same as those used by Salm.[2] The following is a table of the results obtained:

[1] W. B. Hardy, *Journ. of Physiol.*, xxxiii, p. 313, 1905.
[2] E. Salm, *Zeitschr. f. Electrochem.*, x, p. 341, 1904.

TABLE V.

of Cl.	Congo Red.	Cyanin.	Neutral Red.	Tropæolin 000.	Phenolphthalein.	C_{H^+}	Remarks.
0	red	blue violet	orange	pink	red	$>10^{-9}$ N	
1	red	blue violet	orange	pink	red	"	
2	red	blue violet	orange	pink	red	"	
3	red	blue violet	orange	pink	red	"	
4	red	blue violet	orange	pink	red	"	
5	red	blue violet	orange	pink	red	"	
6	red	blue	orange	orange	red	10^{-9} to 10^{-8} N	
7	red	light blue	orange	orange	pink	10^{-9} to 10^{-8} N	
8	red	light blue	orange red	orange	colorless	10^{-8} N	
9	red	light blue	red	orange	colorless	10^{-8} to 10^{-7} N	
10	red	light blue	red	orange	colorless	10^{-8} to 10^{-7} N	Very slight precipitate.
11	red	pale blue	red	orange	colorless	10^{-7} N	Bulky precipitate, filtrate very opalescent.
12	red	very pale blue	red	orange	colorless	10^{-7} to 10^{-6} N	"
13	red	very pale blue	red	orange	colorless	10^{-7} to 10^{-6} N	Bulky precipitate, filtrate fairly clear.
14	brownish red	colorless	red	orange	colorless	10^{-7} to 10^{-5} N	Slight precipitate. solution very unstable; mere filtration or running through a burette and then shaking sufficed to bring down a bulky precipitate which shaking alone did not.
15	brownish red	colorless	red	orange	colorless	10^{-7} to 10^{-5} N	No precipitate.
16	brown ppt., red fluid	colorless	red	reddish orange	colorless	10^{-5} to 10^{-4} N	
17	brown ppt., red fluid	colorless	red	reddish orange	colorless	10^{-5} to 10^{-4} N	Red to litmus.
18	ppt., fluid violet	colorless	magenta	reddish orange	colorless	10^{-4} N	Yellow to rosolic acid.
19	ppt., fluid violet	colorless	magenta	reddish orange	colorless		Reddish orange to methyl orange.
20	ppt., fluid violet	colorless	magenta	reddish orange	colorless		"
21	ppt., fluid violet	colorless	magenta	reddish orange	colorless		"
22	ppt., fluid violet	colorless	magenta	reddish orange	colorless		"
23	ppt., fluid violet	colorless	magenta	reddish orange	colorless		"
24	ppt., fluid violet	colorless	magenta	reddish orange	colorless		"
25	ppt., fluid violet	colorless	magenta	reddish orange	colorless		"

In order to test the influence of salts upon the hydrion concentration in solutions of neutral calcium caseinate, a solution of approximately $\frac{N}{250}$ calcium hydrate was "saturated" with casein and filtered through paper. To 25 c.c. of this solution 5 c.c. of a normal solution of the salt were added and water to make up the volume to 50 c.c. The same proportion of indicator to solution tested was used as in the previous experiments, namely, 0.1 c.c. of a $\frac{M}{100}$ solution of the indicator to 10 c.c. of the solution. The following are the results obtained:

TABLE VI.

Salt.	Congo Red.	Cyanin.	Neutral Red.	Tropæolin 000.	Phenol-phthalein.	C_{H}^{+}
No salt	scarlet	very pale blue	red	orange	colorless	10^{-7} to $10^{-[illegible]}$N
$\frac{N}{10}$ NaCl	"	violet blue	"	"	"	10^{-8} to $10^{-[illegible]}$N
" Na_2SO_4	"	pale blue	"	"	"	10^{-7}N
" KCl	"	"	"	"	"	10^{-7}N
" $NaNO_3$	"	very pale blue	"	"	"	10^{-7} to $10^{-[illegible]}$N
" $LiNO_3$	"	"	"	"	"	10^{-7} to $10^{-[illegible]}$N
" KBr	"	"	"	"	"	10^{-7} to $10^{-[illegible]}$N
" Fe_2Cl_6	brown ppt.	colorless	"	reddish orange	"	10^{-5} to $10^{-[illegible]}$N
" $(COONa)_2$	scarlet	violet blue	"	orange	"	10^{-8} to $10^{-[illegible]}$N
" NH_4NO_3	"	very pale blue	"	"	"	10^{-7} to $10^{-[illegible]}$N
" $KClO_3$	"	"	"	"	"	10^{-7} to $10^{-[illegible]}$N
" Sodium formate	"	pale blue	"	"	"	10^{-7}N
" CH_3COONa	"	"	"	"	"	10^{-7}N
" NH_4CNS	"	blue violet	"	"	"	10^{-8} to $10^{-[illegible]}$N
" Sodium propionate	"	pale blue	"	"	"	10^{-7}N
" Sodium butyrate	"	violet blue	"	"	"	10^{-8} to $10^{-[illegible]}$N
" NaF	"	pale blue	"	"	"	10^{-7}N
" Potassium acetate	"	violet blue	red orange	"	"	10^{-8}N
" KCN	"	blue	orange	"	"	10^{-9} to $10^{-[illegible]}$N

III. HYDROLYSIS OF CASEIN BY TRYPSIN.

(a) *Some Chemical Properties of Trypsin.*

The trypsin used throughout these experiments was that prepared by Grübler according to the directions of Spateholtz, which was very kindly supplied to me by Dr. A. E. Taylor and was the same as that which he used in his experiments on the hydrolysis of protamine.[1]

[1] See A. E. Taylor, *Univ. of Calif. Pub., Pathol.*, i, p. 7, 1904.

It is a pure but not very active product, failing to give the biuret reaction in one per cent. solution, while the ash amounts to only .02 per cent. (Taylor). If one drop of a saturated solution of safranin (Grübler) be added to from 5 to 10 c.c. of a neutral or very faintly alkaline 0.5 per cent. solution of trypsin (neutralized by $\frac{N}{25}$ calcium hydroxide), a light, flocculent, colored precipitate slowly appears on standing and gradually settles. Evidently, in these solutions, the trypsin is acting as an acid and combines with the color-base, safranin, to form an insoluble salt.[1] Confirmatory evidence is not far to seek. Trypsin, in solution, has a distinctly acid reaction; measured by the gas-chain, according to Taylor, a one per cent. solution in carbonic-acid-free water has a hydrogen ion concentration of $\frac{N}{24000}$. I find that 0.5 gram of trypsin dissolved in 100 c.c. of water (not free from carbon dioxide) requires the addition of 2.8 c.c. of $\frac{N}{25}$ calcium hydrate to render the solution neutral to rosolic acid, that is, to bring down the hydrion concentration to from 10^{-6}N to 10^{-7}N.[2]

In addition, Bayliss[3] has recently shown that trypsin, in solution, migrates to the anode under the influence of an electric current, as would be expected were trypsin predominantly acid in character, the colloidal ion being an anion.[4]

The hydrolysis of trypsin itself in acid, alkaline, and even neutral solutions at various temperatures, the influence of the activity of the trypsin preparation upon its rate of destruction, and the influence of proteins, etc., in retarding the destruction have been very carefully dealt with by Vernon,[5] and additional points have been brought out by Bayliss and Starling,[6]

[1] See A. P. Mathews, *Amer. Journ. of Physiol.*, i, p. 445, 1898. Ehrlich, *Farbenanalytische Untersuchungen zur Histologie und Klinik des Blutes*, Berlin, 1891. Gustav Mann, *Physiological Histology*, Oxford, 1902, p. 330, *et seq.*; *Chemistry of the Proteids*, 1906, p. 225. Robertson, this Journal, i, p. 279, 1906.

[2] E. Salm, *loc. cit.*

[3] W. M. Bayliss, *Biochem. Journ.*, i, p. 225, 1906.

[4] Picton and Linder, *Journ. Chem. Soc.*, lxxi, p. 568, 1897. J. Loeb, *Univ. of Calif. Pub., Physiol.*, i, p. 149, 1904.

[5] Vernon, *Journ. of Physiol.*, xxvi, p. 405, 1901; xxviii, p. 386, 1902; xxxi, p. 346, 1904.

[6] Bayliss and Starling, *ibid.*, xxx, p. 61, 1903.

Bayliss,[1] and Taylor.[2] Taylor finds that the optimum alkalinity at which the action of trypsin is most intense, lies within very narrow limits—from $\frac{N}{1110}$ to $\frac{N}{1400}$, using protamine sulphate as substrate.

On dissolving the trypsin in water a small insoluble residue was always left. Taylor finds that filtration deprives the solution of a portion of its activity but secures much more constant results. Bayliss, on the contrary, states that filtration of Grübler's trypsin does not diminish the activity of the solution and concludes that the insoluble residue is not trypsin. In my experiments filtered solutions were used throughout.

(*b*) *The Autohydrolysis of the Caseinates.*

It is a fact of considerable importance, and one upon which I do not think sufficient stress has been laid by previous observers, that the neutral caseinates undergo fairly rapid autohydrolysis in solution. It will be remembered that solutions of the neutral caseinates are practically, if not absolutely, neutral in reaction, so that this fairly rapid hydrolysis is not merely due to the *acceleration* of the autohydrolysis by excess of hydrogen or hydroxyl ions. In several of the sets of experiments upon the hydrolysis of casein by trypsin, controls were made in which the solution of the substrate alone was allowed to digest for a given time and the amount of casein remaining in the solution afterwards determined; in this way several determinations of the velocity constant of the autohydrolysis were made, which, although not usually obtained under strictly comparable conditions of temperature, etc., nevertheless serve to give a general idea of the magnitude and variations of the autohydrolysis. The following table is a summary of the results obtained in this way:

[1] Bayliss, *Arch. des sci. biol.* (St. Petersburg), xi, Suppl., p. 261, 1904, reprinted in the Collected Papers of the University College Physiological Laboratory, London, Vol. xiii.

[2] A. E. Taylor, *Univ. of Calif. Pub., Pathol.*, i, p. 7, 1904.

TABLE VII.

Alkali "Saturated" with Casein.	Mgr. of Casein Initially in Digest.	Time of Digestion in Hours.	Temperature.	Mgr. of Casein Digested.	$\log_{10}\frac{a}{a-x}$ Divided by Time in Hours.
Approx.:					
" $\frac{N}{1500}$ $Ca(OH)_2$	143	3	36.5°	16	17×10^{-3}
" $\frac{N}{1500}$ $Ca(OH)_2$	172	3	37°	16	14×10^{-3}
" $\frac{N}{1250}$ $Ca(OH)_2$	165	3	37°	19	17×10^{-3}
" $\frac{N}{1250}$ $Ca(OH)_2$	168	3	37°	16	14.5×10^{-3}
" $\frac{N}{1000}$ $Ca(OH)_2$	218	3	36.5°	24	17×10^{-3}
" $\frac{N}{800}$ $Ca(OH)_2$	326	3	37°	32	15×10^{-3}

These experiments were all carried out with the unpurified casein as substrate. Thinking that possibly the small quantity of acetanions thus introduced might be in part responsible for this somewhat rapid hydrolysis, I carried out some special experiments with the purified casein. Three determinations of the initial substrate concentration were made, with identical results, while the figures representing the amount digested are the averages of two determinations. The volume of each of the digests was 200 c.c., and a few c.c. of normal sodium chloride were added, just before the acetic acid, to facilitate precipitation.

TABLE VIII.

Alkali "Saturated" with Casein.	Mgr. of Casein Initially in Digest.	Time of Digestion in Hours.	Temperature.	Mgr. of Casein Digested.	$\log_{10}\frac{a}{a-x}$ Divided by Time in Hours.
Approx.:					
" $\frac{N}{2000}$ $Ca(OH)_2$	114	12	38°	33	12×10^{-3}
" $\frac{N}{1333}$ $Ca(OH)_2$	171	12	38°	57	15×10^{-3}

It will be seen that these results are in fair accord with those obtained with the unpurified casein, and we must conclude that neutral calcium caseinate in watery solution undergoes fairly rapid hydrolysis, one third of the substrate being hydrolyzed in twelve hours. I have also observed that neutral sodium caseinate undergoes a similar hydrolysis in watery solution, but I have not determined the actual value of the velocity-constant.

The hydrolysis of neutral caseinates by trypsin, therefore, affords an unusually favorable example, among protein reactions, of the action of an enzyme in *accelerating an already existing reaction.* While, of course, in every case of true catalysis—that is, in every reaction in which the accelerating substance undergoes no diminution during the reaction (or in other words does no work) the catalysor can only accelerate a previously existing reaction[1] (for otherwise work would be gained without expenditure of energy), yet in protein hydrolyses the natural rate of hydrolysis is not usually so great,[2] while the acceleration produced by trypsin is much greater, so that the fact does not stand out so clearly.

(c) Influence of the Concentration of Trypsin upon the Velocity of Hydrolysis.

The older investigators generally failed to find a direct proportionality between the velocity of protein hydrolysis and the concentrations of the ferment. According to Bredig,[3] Mayer, Brücke, Maly, Ellenberger, and Hofmeister found that the velocity of hydrolysis by pepsin was not proportional to the mass of pepsin but that the velocity increased more slowly than the mass of the ferment, so that when a small additional mass of ferment was added to a large mass already present the velocity of hydrolysis was not perceptibly increased. E. Schütz[4] stated that the velocity of peptic digestion of fibrin was proportional to the square root of the mass of the ferment, and Borissoff[5] found that the same rule held good for the digestion of coagulated egg-albumin by trypsin. Pawlow and Walther[6]

1 W. Ostwald, *Lehrbuch der allgemeine Chemie*, ii, Part 2, p. 248, 1902.
2 See, for example, A. E. Taylor, *Univ. of Calif. Pub., Pathol.*, i, p. 49, 1904.
3 Bredig, *Ergebnisse der Physiologie*, i, 1, p. 134, 1902.
4 E. Schütz, *Zeitschr. f. physiol. Chem.*, ix, p. 577, 1895.
5 Borissoff, Thesis, St. Petersburg, 1891, quoted after Vernon, *Journ. of Physiol.*, xxvi, p. 405, 1901.
6 Pawlow-Walther, *Arbeit der Verdauungsdrüsen*, Wiesbaden, 1898, p. 34. quoted after Bredig, *loc. cit.*
Walther, *Arch. des sci. biol.*, vii, p. 15, 1899.

found this rule to hold good for tryptic digestion. Samojiloff[1] found that the velocity of tryptic digestion was only proportional to the square root of the ferment concentration in dilute solutions of ferment; in more concentrated solutions he found that the velocity was less than that required by this rule, while J. Schütz[2] found that the rule held good for the digestion of albumin by trypsin and Vernon[3] confirmed it for the tryptic digestion of swollen fibrin.

In the majority of these investigations the manner of conducting the experiments was such that the processes of solution, diffusion, and hydrolysis were confused, so that the quantities measured bore no necessary physico-chemical relation to the velocity of hydrolysis. The inaccuracies dependent upon the use of solid protein and particularly of Mett's method have been dealt with by Taylor,[4] who has drawn attention to the fact that the velocities of diffusion and of solution both play a part in determining the results obtained by these methods. Moreover, in the majority of cases, no precautions were taken to control the alkalinity of the digests, and the alkalinity, when alluded to, is usually somewhat vaguely expressed in terms of the concentration of sodium carbonate, without mention of the concentration of carbon dioxide already present in the digest. With indeterminate and possibly varying alkalinity, with continual destruction of the ferment by the most unnecessarily high alkalinities used,[5] and the use as a measure of the velocity of hydrolysis of a quantity involving other and quite different factors, it is not surprising that relations between ferment concentration and velocity of hydrolysis were found which did not readily admit of physico-chemical interpretation, although Hofmeister[6] endeavored to account for the Schütz rule by the dissociation of the ferment according to the dilution-law.

More recent investigators, working with more refined methods under more exactly controlled and defined conditions, agree in

[1] Samojiloff, *ibid.*, ii, p. 699.
[2] J. Schütz, *Zeitschr. f. physiol. Chem.*, xxx, p. 1, 1900.
[3] Vernon, *Journ. of Physiol.*, xxvi, p. 405, 1901.
[4] A. E. Taylor, *Univ. of Calif. Pub., Pathol.*, i, p. 7, 1904.
[5] See Vernon, *loc. cit.*
[6] Hofmeister, quoted after Bredig, *loc. cit.*

stating that within certain limits of substrate and hydrion concentration, temperature, etc., the velocity of protein hydrolysis is directly proportional to the mass of the ferment, as would be expected if the enzyme acted upon the substrate through the formation of an intermediate compound which breaks down, regenerating the ferment. This has been shown to hold good for pepsin by Sjöqvist[1] and by Sawjalow,[2] for trypsin by V. Henri and Larguier des Bancels,[3] working with gelatin as substrate, by A. E. Taylor[4] working with protamine as substrate, and by Bayliss,[5] working with casein as substrate, and for erepsin by Vernon.[6]

In the experiments upon trypsin, V. Henri and Larguier des Bancels and Bayliss used the increase in electrical conductivity as a measure of the amount of substrate digested, while Taylor used a direct method, namely, that of measuring the time which elapses until the solution of protamine sulphate ceases to give a precipitate with acidulated alcohol.

The direct proportionality does not hold good with exactitude at high substrate concentration (Taylor), at a sufficiently high hydroxidion concentration to destroy an appreciable amount of the ferment in the time occupied by digestion, or in the later stages of the reaction (Bayliss).

The exact and extensive investigations to which I have referred really suffice to establish the law of direct proportionality, under optimal conditions, for the substrates employed. In Bayliss' experiments with casein, however, the amount digested is measured indirectly by means of the increase in conductivity, involving the assumption that the increase in conductivity is proportional to the amount digested, so that it appeared to me that it would be satisfactory to repeat the

1 Sjöqvist, *Skand. Arch. f. Physiol.*, v, p. 277, 1895.

2 Sawjalow, *Zeitschr. f. physiol. Chem.*, xlvi, p. 307, 1905.

3 V. Henri and Larguier des Bancels, *Compt. rend. de l'Acad. des sci.*, cxxxvi, pp. 1099 and 1581, 1903.

4 A. E. Taylor, *loc. cit.*

5 Bayliss, *Arch. des sci. biol.*, xi, Suppl., p. 261, 1904; reprinted in the Collected Papers of the Physiological Laboratory, University College, London, Vol. xiii.

6 Vernon, *Journ. of Physiol.*, xxx, p. 334, 1903.

experiments, using the direct method of estimation which I have described. Moreover, I was unable to find in the literature any statement as to whether the valency of the cation of the alkali combined with the casein influenced the relation between the velocity of digestion and the concentration of the trypsin, a point which, for theoretical reasons, it appeared to me advisable to settle. In all cases the neutral calcium caseinate was employed as substrate, so that the reaction of the digest initially was practically that of conductivity water. The digestions were carried out in air-tight flasks so that no carbon dioxide could enter except the trifling amount already present in the air in the flask. As I will show later on, the hydrion concentration does not alter perceptibly in an initially neutral digest as digestion proceeds, at least for the periods in question, although the reactions of initially acid or alkaline digests tend to neutrality as digestion proceeds. Thus no appreciable error was introduced in these experiments on account of varying hydrion or hydroxidion concentration. The substrate used by Bayliss was sodium caseinate, so I confined my experiments in this direction to calcium and barium caseinates. The digests were all carried out in Erlenmeyer flasks of 500 c.c. capacity; these flasks had initially been washed with strong sulphuric acid and thoroughly rinsed out and then stood in several changes of tap-water for over a fortnight to get rid of the last traces of sulphuric acid: they were then rinsed again several times with tap- and finally distilled water: the same flasks were used throughout in the digestion experiments, being thoroughly washed out after each set of experiments and stood for at least twelve hours in tap-water before beginning a fresh set, in order to allow bacterial digestion to get rid of any last traces of protein material which might possibly remain adhering to the glass; they were then rinsed in tap- and distilled water as before. The flasks were closed with rubber stoppers which had been boiled and finally steamed for several hours and then washed in tap- and distilled water; these stoppers always stood under water between digests and were washed in distilled water just before use. Such precautions were found to be necessary by Taylor, the slightest cause, such as the use of vulcanized rubber for stoppers, excessively delaying the reaction. Toluene (Kahlbaum) was used

to keep the digress sterile as this has a negligible influence on the velocity of the reaction for short experiments.[1] The casein used was not purified, but the slight trace of acetanions present, as we have seen, does not appreciably alter the reaction of the solution of the neutral caseinate or the velocity of autohydrolysis.

Experiment 1.—Ten grams of casein were shaken up in five liters of approximately $\frac{N}{1000}$ $Ca(OH)_2$, the suspension was filtered once through glass wool and then once through paper.[2] Five c.c. of toluene were added, the mixture shaken, and 200 c.c. measured out into each flask. A 0.05 per cent. solution of the trypsin was made up and filtered through S and S, 589, "black band" filter-paper and 0.5, 1, 2, 3, and 4 c.c. of this solution added to the digests. The experiments were done in duplicate. The amount of casein initially in 200 c.c. was determined in the usual way and found to be 0.218 grams. The digestion was carried out at 36.5° C. for three hours, at the end of which time the casein in all the digests was precipitated by acetic acid and estimated as usual.

If the law, $\log \frac{a}{a-x} = Kt$, for a monomolecular reaction, holds good and K is proportional to the concentration of the ferment, we should, after a given interval of time, find $\log \frac{a}{a-x}$ proportional to the number of cubic centimeters of trypsin solution which had been initially added to the digest, a being the initial concentration of the substrate and x the amount of casein digested in the given interval of time, provided, that is, that the ferment did not decay to an appreciable extent during the digestion and that, at the interval of time chosen, the products of the reaction were not yet present in sufficient amount to appreciably depress the velocity of the reaction. The following were the results obtained:

1 Bayliss, *loc. cit.* Kaufmann, *Zeitschr. f. physiol. Chem.*, xxxix, p. 452, 1903.

2 If filtered in the first place through paper the filtration is slow. If filtered only through lightly packed glass wool the separation of the undissolved particles is apt to be incomplete. Time is saved by the double procedure outlined above.

TABLE IX

C.C. of Trypsin Solution.	Milligrams of Casein Digested.	$\log_{10}\frac{a}{a-x}$	$\log_{10}\frac{a}{a-x}$ Divided by Number of c.c. of Trypsin Solution.
0.5	16	.033105	66×10^{-3}
1	32	.068943	69×10^{-3}
2	54	.123612	63×10^{-3}
3	78	.192328	64×10^{-3}
4	101	.270270	67.5×10^{-3}

The figures in the second column are each the average of two determinations. The figures in the last column are approximately constant, so that we may conclude that at this substrate concentration the velocity of hydrolysis at any instant is directly proportional to the concentration of the trypsin.

Experiment 2.—Procedure precisely as in last experiment except that $\frac{N}{500}$ calcium hydrate was used in place of $\frac{N}{1000}$ calcium hydrate "saturated" with casein; the concentrations of ferment used were 1, 2, 3, 4, and 5 c.c. of a 0.05 per cent. solution. The initial amount of casein in each digest (volume 200 c.c.) was 320 milligrams, this being the mean of three determinations. The digestion was carried out at 36° C. for three hours. The following were the experimental results:

TABLE X

C.C. of Trypsin Solution.	Milligrams of Casein Digested.	$\log_{10}\frac{a}{a-x}$	$\log_{10}\frac{a}{a-x}$ Divided by Number of c.c. of Trypsin Solution.
1	46	.067399	67×10^{-3}
2	68	.103749	52×10^{-3}
3	107	.176770	59×10^{-3}
4	130	.226396	56.5×10^{-3}
5	178	.352862	70.5×10^{-3}

The figures in the second column are each the mean of three determinations, except the second and the fifth, each of which is based on one determination only. The figures in the last column are again fairly constant, so that at this substrate concentration also the rapidity of digestion is proportional to the concentration of the ferment. Moreover the constant is practically the same in the two cases; the values in this experiment

are as a rule slightly lower than in the last but the difference of 0.5° in temperature would account for part of this difference.

Experiment 3.—Precisely as in the two previous experiments except that $\frac{N}{100}$ barium hydrate was used in place of calcium hydrate "saturated" with casein. The concentrations of ferment used were 1, 1.5, 2, 2.5, 3, and 5 c.c. of a 0.05 per cent. solution in digests of 200 c.c. volume. The initial amount of casein in each digest was 238 milligrams (the mean of two determinations). The digestion was carried out at 35.5° C. for three hours. The following are the experimental results:

TABLE XI

C.C. of Trypsin Solution.	Milligrams of Casein Digested.	$\log_{10} \frac{a}{a-x}$	$\log_{10} \frac{a}{a-x}$ Divided by Number of c.c. of Trypsin Solution.
1	20	.038121	38×10^{-3}
1.5	28	.054358	36×10^{-3}
2	39	.077724	39×10^{-3}
2.5	46	.093276	37×10^{-3}
3	52	.107064	36×10^{-3}
5	93	.215209	43×10^{-3}

The figures in the second column are the averages of two determinations each except the last, in which case only one determination was made. It will be seen that, as in the other two experiments, the figures in the last column are constant, so that the velocity of hydrolysis is proportional to the concentration of the trypsin. In this case the constant is only two-thirds the magnitude of the constant for calcium caseinate, showing that barium caseinate is only digested with about two thirds the rapidity at which an equivalent concentration of calcium caseinate is digested under similar conditions. It should be noted, however, that the temperature in this experiment was 1° lower than in Experiment 1, so that at least 10 per cent. of their value should be added to each of the figures in the last column of Table XI in order to compare with those in the last column of Table IX.

Experiment 4.—Procedure precisely as in the previous experiments, except that $\frac{N}{400}$ calcium hydrate "saturated" with casein was used. The concentrations of ferment used were 1, 2, 4, and 6 c.c. of a 0.1 per cent. solution. The initial amount

of calcium caseinate in each digest was 431 milligrams. The digestion was carried out at 36° C. for three hours. The following are the experimental results:

TABLE XII

C.C. of Trypsin Solution.	Milligrams of Casein Digested.	$\mathrm{Log}_{10}\frac{a}{a-x}$	$\mathrm{Log}_{10}\frac{a}{a-x}$ Divided by Number of c.c. of Trypsin Solution.
1	49	.052414	52×10^{-3}
2	109	.126621	63×10^{-3}
4	232	.335624	84×10^{-3}
6	336	.656753	109×10^{-3}

The figures in the second column are based on one determination each. It will be observed that the figures in the last column depart widely from a constant value; in fact if we denote the ratio of log $\frac{a}{a-x}$ to the number of c.c. of ferment solution by y we find that $y = 41 + 11f$ where f is the concentration of the ferment. Comparing the observed values with those calculated from the above formula, we have:

C.C. of Trypsin	1	2	4	6
y (calculated)	52	63	85	107
y (observed)	52	63	84	109

Hence at this substrate concentration the velocity of digestion was proportional to $41f + 11f^2$ where f is the ferment concentration.

The theoretical bearings of this formula will be discussed later, but meanwhile we may point out the analogy with the formula found by Arrhenius[1] connecting the velocity of the hydrolysis of sugar with the hydrion concentration *at high concentrations of acid.*

(d) *Relation between the Amount Hydrolysed and the Time of Hydrolysis by Trypsin.*

V. Henri and Larguier des Bancels[2] found that the formula for a monomolecular reaction, log $\frac{a}{a-x} = Kt$, where a is the initial

[1] Arrhenius, *Zeitschr. f. physikal. Chem.*, iv, p. 244, 1889.

[2] V. Henri and Larguier des Bancels, *Compt. rend. de l'Acad. des sci.*, cxxxvi, pp. 1099, 1581, 1903.

substrate concentration, x is the amount digested, and t is the time, expressed the rate of hydrolysis of gelatin by trypsin, and Bayliss[1] confirmed this formula for the earlier stages of the digestion of sodium caseinate, while in the later stages the reaction was delayed, either owing to the accumulation of the products of hydrolysis or else to the gradual decay of the trypsin. Both Henri and Larguier des Bancels and Bayliss used the conductivity method.

In connection with other experiments I made a few observations on the relation between the time of hydrolysis and its extent. A solution of approximately $\frac{N}{100}$ calcium hydrate was "saturated" with casein and 50 c.c. placed in Erlenmeyer flasks of 100 c.c. capacity which had been well steamed and were tightly closed with rubber stoppers which had also been well steamed and boiled. One twentieth of a cubic centimeter of toluol was added to each flask, 1 c.c. of a 0.025 per cent. solution of trypsin added, and the solutions digested at 37.5°± .25°. The initial amount of casein in 50 c.c. was 132 milligrams. The following were the results obtained:

TABLE XIII

Time in Hours.	Milligrams of Casein Digested.	$\log_{10}\frac{a}{a-x}$	$\log_{10}\frac{a}{a-x}$ Divided by Time in Hours.
1.5	15	.052388	35×10^{-3}
3	27	.099385	33×10^{-3}
4.5	32	.120574	27×10^{-3}
6	46	.186076	31×10^{-3}

The figures in the second column are each the mean of two determinations. The results are in fair accord with the formula $\log\frac{a}{a-x}=Kt$, values for the quotient of $\log\frac{a}{a-x}$ by the time remaining practically constant. These figures, however, only represent the course of digestion in the early stages, as only about one-third of the total casein present had been digested when the observations were discontinued.

[1] Bayliss, *loc. cit.*

(e) *The Influence of Salts upon the Hydrolysis of Casein by Trypsin.*

A large number of investigations have been published on the influence of dissolved substances upon the action of trypsin, but since the majority of these investigations appear to have been carried out under the impression, which at one time prevailed among biologists, that solutions containing equal *percentages* of the various substances studied were solutions of comparable concentration, and since, as we shall see, the influence which a salt exerts upon the rate of hydrolysis by trypsin bears no simple relation to its concentration, the results obtained by these observers afford few or no data regarding the comparative efficiency of salts, etc., in accelerating or retarding the hydrolysis.

According to Oppenheimer,[1] the first to systematically study the action of "neutral salts" upon the action of trypsin was Podolinski,[2] who found that *all* salts promote the action of trypsin but that the intensity of this influence varies greatly. As early as 1872, however, Schaefer and Boehm[3] stated that arsenious acid had no effect on tryptic activity, and in 1875 Heidenhain[4] pointed out that sodium chloride accelerated the action of trypsin. Lindberger[5] found that the inhibitory action of many organic acids could be partly overcome by bile plus sodium chloride, but that in general, while sodium chloride accelerated in neutral or alkaline solutions, in acid solutions sodium chloride retarded the action of trypsin, nor was this retardation due to destruction of the ferment, for after dialysis the ferment could be recovered in an active condition. Bubnow[6] found that ferric chloride and ferrous sulphate had no action on trypsin. Pfeiffer[7] came to a conclusion diametrically opposed to that of Podolinski, namely, that with the exception of sodium

[1] Oppenheimer, *Ferments and their Actions*, trans. by Mitchell, 1901, p. 108.

[2] Podolinski, *Beitr. z. Kenntn. d. pancr. Eiweissverdg.*, Inaug. Diss., Breslau, 1876, quoted after Oppenheimer, *loc. cit.*

[3] Schaefer and Boehm, *Jahresber. f. Thierchem.*, p. 367, 1872.

[4] Heidenhain, *Arch. f. d. ges. Physiol.*, x, p. 579, 1875.

[5] Lindberger, *Jahresber. f. Thierchem.*, p. 282, 1883.

[6] Bubnow, *Zeitschr. f. physiol. Chem.*, vii, p. 315, 1883.

[7] Pfeiffer, *Jahresber. f. Thierchem.*, p. 278, 1884.

carbonate all salts, especially sodium chloride, *retard* tryptic digestion. Harris and Tooth[1] found that mercuric chloride delays the action of Liquor Pancreaticus but does not abolish it, while Wasiliew[2] had previously stated that trypsin was unaffected by mercuric chloride.

Chittenden and Cummins[3] published an extensive series of experiments dealing with the influence of salts on tryptic digestion in which they investigated the influence exerted by a variety of concentrations. Unfortunately, however, they used dried fibrin as a substrate, in neutral solution, and weighed the undigested residue after six hours' digestion at 40° C. Here, as in Mett's method and all methods in which an insoluble substrate is used, the processes of solution, diffusion, and digestion combine, as I have pointed out, to produce the result. They found, however, that mercuric chloride, mercuric idodide, mercuric bromide, mercuric cyanide, cupric sulphate, lead acetate, stannous chloride, arsenious oxide, arsenic acid, potassium antimony tartrate, ferric chloride, ferrous sulphate, manganous chloride, zinc sulphate, barium chloride, magnesium sulphate, potassium permanganate, potassium bichromate, potassium ferrocyanide, potassium ferricyanide, sodium sulphate, potassium chlorate, potassium nitrate, and potassium chloride all retarded the hydrolysis to a greater or less extent, while potassium cyanide, in concentrations of 0.1 per cent. (corresponding to $\frac{N}{65}$) and upwards, produced *marked acceleration*, and sodium tetraborate also accelerated the digestion although to a less degree. Sodium chloride accelerated at 0.05 per cent. (corresponding to $\frac{N}{117}$) and retarded at higher concentrations, while the action of potassium bromide and of potassium iodide was in the direction of acceleration.

More recently Weiss[4] has found that sodium chloride delays tryptic digestion in strong concentrations although in very weak concentrations (0.05 per cent.) it accelerates. A 10 per cent. sodium chloride solution, according to Weiss, retards digestion to

[1] Harris and Tooth, *Journ. of Physiol.*, ix, p. 223, 1888.
[2] Wasiliew, *Zeitschr. f. physiol. Chem.*, vi, p. 112, 1882.
[3] Chittenden and Cummins, *Trans. Connecticut Acad.*, vii, p. 108.
[4] Weiss, *Zeitschr. f. physiol. Chem.*, xl, p. 480, 1904.

the extent of 13 per cent., while sodium bromide and sodium iodide act more weakly. Potassium salts, he states, act similarly, borax favors the reaction, oxalates and sulphates strongly inhibit it, and disodium phosphate accelerates the digestion.

In regard to the influence of reaction very contradictory statements are found in the older literature. It was early recognized that alkalies and salts, such as sodium carbonate, possessing an alkaline reaction accelerated the action of trypsin, but the first to recognize the really active agent in this acceleration appear to have been Dietz and Kanitz [1] who found that the velocity of digestion was proportional to the hydroxyl concentration, the latter placing the optimum alkalinity at a concentration of $\frac{N}{400}$ hydroxidion. That entirely unnecessary and excessive concentrations of alkali were used by the majority of the older investigators was pointed out by Vernon [2] who found that destruction of the ferment occurred in distilled water, the destruction in strong extracts sometimes amounting to 33 per cent. within an hour, while in weaker extracts destruction took place much more slowly; this destruction is very much accelerated by hydroxyl ions. Bayliss and Starling [3] confirmed these observations of Vernon while Taylor,[4] using protamine sulphate as substrate, found that the optimum hydroxyl concentration lay between $\frac{N}{1110}$ and $\frac{N}{1400}$. The rate of digestion is, however, not simply a function of hydroxyl concentration, for rapid hydrolysis takes place in the presence of 0.4 per cent. sodium carbonate corresponding to an hydroxyl concentration which, on direct addition, inhibits digestion[5]; and the optimum sodium carbonate concentration given by Vernon is 0.5 per cent., corresponding to $\frac{N}{200}$ hydroxidion, a degree of alkalinity which, according to Taylor, sufficed in his experiments to inhibit tryptic digestion entirely. Bayliss and Starling [6] and previously

[1] Kanitz, *Zeitschr. f. physiol. Chem.*, xxxvii, 1902, who also quotes Dietz.

[2] Vernon, *Journ. of Physiol.*, xxviii, p. 386, 1902.

[3] Bayliss and Starling, *ibid.*, xxx, p. 61, 1903.

[4] Taylor, *Univ. of Calif. Pub., Pathol.*, i, p. 7, 1904.

[5] Taylor, *loc. cit.*

[6] Bayliss and Starling, *Journ. of Physiol.*, xxx, p. 61, 1903.

Biernacki[1] found that the addition of dissolved proteins to an alkaline solution containing trypsin greatly retarded the destruction of the trypsin, and Vernon[2] pointed out that the protective power of these substances for trypsin depends solely upon their power of neutralizing alkali and if their acid radicles be previously neutralized by alkali they lose their protective power. Most proteins have some protective value but hydrated proteins and the decomposition products of protein hydrolysis have greater protective value. In some cases, however, there is apparently a combination between the ferment and the protein which only breaks down very slowly; thus egg-albumin possesses marked antitryptic properties.[3]

In regard to the influence of other dissolved substances, Fraser[4] found that watery infusions of tea, coffee, and cocoa retard tryptic digestion, particularly the digestion of casein. This retardation he afterwards attributed to the tannic acid contained in these infusions.[5] Heidenhain[6] found that bile and sodium glycocholate accelerated the hydrolysis, but Chittenden and Albro[7] found that bile did not accelerate tryptic digestion, while Rachford[8] stated the contrary to be the case.

Bruno[9] and Zuntz[10] confirmed Rachford's results, but Vernon[11] finds that at the optimum alkalinity bile has no influence on tryptic digestion, but if the concentration of the bile is as high as 10 to 40 per cent. it retards.

1 Biernacki, *Zeitschr. f. Biol.*, xxviii, p. 55, 1891.
2 Vernon, *Journ. of Physiol.*, xxxi, p. 346, 1904.
3 See also Bayliss, *Arch. des sci. biol.*, xi, Suppl., p. 261, 1904. Reprinted in the Collected Papers of the Physiological Laboratory, University College, London, vol. xiii.
4 Fraser, *Journ. of Anat. and Physiol.*, xxi, p. 337, 1887.
5 Fraser, *ibid.*, xxxi, p. 469, 1897.
6 Heidenhain, *Arch. d. ges. Physiol.*, x, p. 579, 1875.
7 Chittenden and Albro, *Amer. Journ. of Physiol.*, i, p. 307, 1898.
8 Southgate and Rachford, *Med. Rec.*, p. 878, 1895. Rachford, *Journ. of Physiol.*, xxv, p. 165, 1899.
9 Bruno, *Arch. des sci. biol. de St. Petersbourg*, vii, p. 14, quoted after Hammarsten, *Text-book of Physiol. Chem.*, trans. by Mandel, 1904, p. 328.
10 Zuntz, *Arch. f. Anat. u. Physiol.*, p. 380, 1900.
11 Vernon, *Journ. of Physiol.*, xxviii, p. 386, 1902.

Kaufmann [1] finds that toluol, chloroform, thymol, and sodium fluoride do not retard tryptic digestion by a 0.2 or higher per cent. solution of Grübler's trypsin. Weaker trypsin solutions are retarded by these antiseptics, the more the weaker the trypsin solution. After twenty-four hours a retarding action on a 0.08 per cent. solution of trypsin under the influence of toluol could be observed, sodium fluoride weakened the ferment of 0.06 per cent. concentration, chloroform a solution of 0.08 per cent. concentration, and thymol a solution of 0.1 per cent. concentration. Bayliss [2] has confirmed Kaufmann's results as far as toluol is concerned.

Bräunnung [3] finds that tryptic digestion is retarded by glycerin, cane sugar, and gum arabic, whereas Weiss [4] finds that gum arabic favors tryptic digestion.

Price [5] finds that the addition of formalin in the proportions of 1 to 10,000 and 1 to 20,000 does not alter the digestibility of milk by trypsin.

In regard to alkaloids, Chittenden and Cummins [6] find that the sulphates of morphin, atropin, strychnin, brucin, narcotin, quinin, cinchonin, and cinchonidin all retard tryptic digestion in a greater or less degree; on the other hand Wroblewski [7] finds that caffein, nicotin, and conin accelerate the hydrolysis, atropin does so slightly, and morphin retards slightly and veratrin retards strongly.

In regard to dissolved gases, Chittenden and Cummins [8] find that carbon dioxide and hydrogen sulphide retard tryptic digestion in neutral solutions, while hydrogen accelerates slightly. According to Schierbeck,[9] however, carbon dioxide accelerates in a faintly alkaline solution while it retards in an

[1] Kaufmann, *Zeitschr. f. physiol. Chem.*, xxxix, p. 434, 1903.

[2] Bayliss, *loc. cit.*

[3] Bräunnung, *Zeitschr. f. physiol. Chem.*, xlii, p. 70, 1905.

[4] Weiss, *ibid.*, xl, p. 480, 1904.

[5] Price, *Centralbl. f. Bact.*, xiv (2), Nos. 3 and 4, 1905, quoted after *Biochem. Centralbl.*, iii, p. 722.

[6] Chittenden and Cummins, *loc. cit.*

[7] Wroblewski, *Zeitschr. f. physiol. Chem.*, xxi, p. 1, 1895.

[8] Chittenden and Cummins, *loc. cit.*

[9] Schierbeck, *Skand. Arch. f. Physiol.*, iii, p. 344, 1892.

acid solution. Probably the acceleration obtained by Schierbeck in alkaline solution was due to the partial neutralization of an injurious excess of alkali.

In regard to the influence of other ferments many contradictory statements have been made regarding the influence of pepsin upon the activity of trypsin. In the older literature pepsin is generally stated to have a destructive action on trypsin,[1] but these effects seem mainly to have been due to the acid reaction communicated to the digest by the addition of acid solutions of pepsin, for Fischer and Abderhalden,[2] as might have been expected, found that by the combined action of pepsin and trypsin on casein they secured a more intense hydrolysis.

It is obvious that the evidence concerning the influence of dissolved substances, and particularly of salts, upon the hydrolysis of protein is of a highly unsatisfactory and contradictory nature, while the experiments with salts have rarely been carried out under adequate conditions of physico-chemical control. I therefore proceeded to carry out a series of experiments on the influence of salts upon the hydrolysis of neutral calcium caseinate by trypsin. In all cases the volume of the digest was 200 c.c. and at the end of digestion the casein was precipitated by acetic acid and estimated in the usual way. The results obtained are given in Table XIV, the figures in the last two columns being the values of K in the equation $\log \frac{a}{a-x} = Kt$ for the control digest containing the ferment alone and for that containing the ferment plus salt respectively.

These experiments were not all carried out under comparable conditions of temperature, substrate concentration, ferment concentration, etc., but the following groups of salts (page 364) were tested simultaneously under exactly comparable conditions. The salts are placed in the order in which they accelerate hydrolysis, the most powerful accelerators being placed first.

[1] Mays, *Untersuch. Physiol. Instit. Heidelberg*, iii, p. 378, quoted after Oppenheimer, *Ferments and Their Actions*, trans. by Mitchell, 1901, p. 108. J. N. Langley, *Journ. of Physiol.*, iii, p. 263, 1882. Kühne, *Verh. Nat. Med. Ver.*, Heidelberg, 1877, p. 190, quoted after Oppenheimer, *loc. cit.*

[2] Emil Fischer and Abderhalden, *Zeitschr. f. physiol. Chem.*, xl, p. 215. 1903.

TABLE XIV

Salt	Alkali "Saturated" with Casein	Temperature.	Time in Hours.	Milligrams of Casein Originally in Digest.	Concentration of Ferment.	Milligrams Digested by Ferment plus Salt.	K (Ferment alone.)	K_1 (Ferment plus Salt.)
.05N NaCl	Approx. $\frac{N}{400}$ NaOH	33°	12	479	1 c.c. .1%	373	45	67
.05N "	" $\frac{N}{1600}$ $Ca(OH)_2$	36.5°	3	143	1 c.c. .025%	49	11	44
.1N "	" $\frac{N}{1333}$ $Ca(OH)_2$	37°	3	172	1 c.c. .1%	77	62	69
.125N "	" " "	37°	3	172	1 c.c. .1%	83	62	78
.15N "	" " "	37°	3	172	1 c.c. .1%	80	62	74
.175N "	" " "	37°	3	172	1 c.c. .1%	83	62	78
.05N KCl	" $\frac{N}{400}$ NaOH	33°	12	479	1 c.c. .1%	365	45	62
.05N "	" $\frac{N}{1600}$ $Ca(OH)_2$	36.5°	3	143	1 c.c. .025%	46	11	39
.175N "	" $\frac{N}{1333}$ $Ca(OH)_2$	37°	3	172	1 c.c. .1%	96	62	101
.025N RbCl	" $\frac{N}{1000}$ $Ca(OH)_2$	36°	3	169	1 c.c. .05%	81	41	77
.025N CsCl	" " "	36°	3	169	1 c.c. .05%	66	41	55
.05N $CaCl_2$	" $\frac{N}{1600}$ $Ca(OH)_2$	36.5°	3	143	1 c.c. .025%	51	11	47
.175N "	" $\frac{N}{1333}$ $Ca(OH)_2$	37°	3	172	1 c.c. .1%	86	62	83
.05N $NaNO_3$	" $\frac{N}{400}$ NaOH	33°	12	479	1 c.c. .1%	351	45	55
.05N NH_4NO_3	" " "	33°	12	479	1 c.c. .1%	360	45	60
.05N KBr	" " "	—	12	558	1 c.c. .1%	468	49	60
.05N Na_2SO_4	" $\frac{N}{1600}$ $Ca(OH)_2$	36.5°	3	143	1 c.c. .025%	41	11	32
.175N "	" $\frac{N}{1333}$ $Ca(OH)_2$	37°	3	172	1 c.c. .1%	92	62	94
.025N NaF	" $\frac{N}{1000}$ $Ca(OH)_2$	36°	3	169	1 c.c. .50%	78	41	73
.025N NaH_2PO_4	" $\frac{N}{1000}$ $Ca(OH)_2$	36°	3	169	1 c.c. .05%	25	41	6
.025N Na_2HPO_4	" " "	36°	3	169	1 c.c. .05%	nearly 169	41	very large

TABLE XIV (*continued*)

Salt.	Alkali "Saturated" with Casein.	Tempera-ture.	Time in Hours.	Milligrams of Casein Originally in Digest.	Concentration of Ferment.	Milligrams Digested by Ferment plus Salt.	K (Ferment alone.)	K_1 (Ferment plus Salt.)
.05N NH_4CNS	Approx. $\frac{N}{400}$ NaOH	—	12	558	1 c.c. .1%	449	49	53
.05N "	" $\frac{N}{1600}$ $Ca(OH)_2$	36.5°	3	143	1 c.c. .025%	51	11	47
.175N "	" $\frac{N}{1333}$ $Ca(OH)_2$	37°	3	172	1 c.c. .1%	67	62	54
.025N KCN	" $\frac{N}{1000}$ $Ca(OH)_2$	36°	3	169	1 c.c. .05%	nearly 169	41	very large
.05N Sodium formate	" $\frac{N}{400}$ NaOH	—	12	558	1 c.c. .1%	491	49	71
.175N Sodium formate	" $\frac{N}{1333}$ $Ca(OH)_2$	37°	3	172	1 c.c. .1%	116	62	145
.05N CH_3COONa	" $\frac{N}{400}$ NaOH	33°	12	479	1 c.c. .1%	388	45	78
.05N "	" $\frac{N}{1600}$ $Ca(OH)_2$	36.5°	3	143	1 c.c. .025%	nearly 143	11	very large
.05N CH_3COOK	" $\frac{N}{400}$ NaOH	—	12	558	1 c.c. .1%	494	49	72
.05N Sodium propionate	" " "	33°	12	479	1 c.c. .1%	408	45	102
.05N Sodium butyrate	" " "	—	12	558	1 c.c. .1%	494	49	72
.05N Sodium valerianate	" " "	—	12	558	1 c.c. .1%	504	49	79
.025N Sodium oxalate	" $\frac{N}{1000}$ $Ca(OH)_2$	36°	3	169	1 c.c. .05%	94	41	101
.025N Sodium tartrate	" " "	36°	3	169	1 c.c. .05%	75	41	66

TABLE XIV (*continued*)

Salt.	Alkali "Saturated" with Casein.	Temperature.	Time in Hours.	Milligrams of Casein Originally in Digest.	Concentration of Ferment.	Milligrams Digested by Ferment plus Salt.	K (Ferment alone.)	K_1 (Ferment plus Salt.)
.025N Sodium citrate	Approx. $\frac{N}{1000}$ $Ca(OH)_2$	36°	3	169	1 c.c. .05%	nearly 169	41	very large
.025N $CuCl_2$	" " "	36°	3	169	1 c.c. .05%	nearly 169*	41	very large*
.025N Fe_2Cl_6	" " "	36°	3	169	1 c.c. .05%	nearly 169*	41	very large*

* There are indications that Fe_2Cl_6 and, possibly, $CuCl_2$ prevent the precipitation by acetic acid; too much reliance must not, therefore, be placed on the results for these salts. With .025N $HgCl_2$ a precipitate was obtained by the addition of acetic acid, but it did not dissolve again in alkali and so could not be estimated.

I.

Sodium propionate
Sodium acetate
Sodium chloride
Potassium chloride
Ammonium nitrate
Sodium nitrate

II.

Sodium valerianate
Potassium acetate
Sodium butyrate
Sodium formate
Potassium bromide
Ammonium sulphocyanate

III.

Sodium acetate
Ammonium sulphocyanate
Sodium chloride
Potassium chloride
Sodium sulphate

IV.

Sodium formate
Potassium chloride
Sodium sulphate
Sodium chloride
Ammonium sulphocyanate

V.

Potassium cyanide
Sodium citrate
Disodium phosphate
Sodium oxalate
Rubidium chloride
Sodium fluoride
Sodium tartrate
Cæsium chloride
Monosodium phosphate (retards)

There is a striking correspondence between the order in which the above salts accelerate the hydrolysis of neutral caseinates by trypsin and the order in which they augment the solubility of casein in calcium hydrate (see Table IV). Since the curve showing the relation between the concentration of the salt and the degree of acceleration which it produces exhibits, as we shall see, marked maxima and minima it is not improbable that the curves for different salts not too far removed from one another in respect to their influence upon the velocity of hydrolysis may cut one another, so that at one concentration the salts may accelerate in one order and at another concentration in another order; a comparison of Groups III and IV shows this clearly. Nevertheless we may state broadly, classifying the ions according to their anions, that potassium cyanide accelerates the tryptic hydrolysis of casein most, then follow the salts of the fatty acids (valerianate, butyrate, acetate, etc.), while chlorides, nitrates, and sulphates accelerate least. This is an order not commonly

found in investigations upon the influences of salts on protein systems. It exhibits marked divergences from that given by Pauli [1] for the coagulation of egg-white by salts, nor is it in better agreement with the order in which salts influence the swelling of gelatin in water, as given by Hofmeister.[2] But it is in fair agreement with the order in which salts dissolve serum globulin, as found by Hardy,[3] and still better, as I have pointed out, with the order in which they augment the solvent power of calcium hydrate for casein.

We may, I think, conclude from the above data that *those salts which augment most the solvent power of calcium hydrate for casein and which diminish to the greatest extent the opalescence of solutions of calcium caseinate also accelerate to the greatest extent the hydrolysis of calcium caseinate by trypsin.*

(f) *The Influence of the Degree of Alkalinity upon the Acceleration in the Hydrolysis of Casein by Trypsin which is Brought about by the Addition of Sodium Chloride.*

Two hundred c.c. of an approximately $\frac{N}{1000}$ calcium hydrate solution "saturated" with casein was placed in each flask, and 0, 3, 6, and 9 c.c. of $\frac{N}{25}$ calcium hydrate added and the volume of each digest was made up to 230 c.c. The initial mass of casein in the digests was determined in the usual way and found to be 187 milligrams. Digestion was carried on at 36° C. for three hours. The following tables express the result.

TABLE XV.

(without ferment)

C.C. of $\frac{N}{25}$ $Ca(OH)_2$.	K (without NaCl)	K_1 (plus NaCl)	K_1 when $K=1$.
0	17	30	1.77
3	23	45	1.96
6	30	49	1.63
9	39	49	1.26

[1] W. Pauli, *Beitr. z. chem. Physiol. u. Path.*, iii, p. 225, 1903.
[2] Hofmeister, *Arch. f. exper. Path. u. Pharm.*, xxvii, p. 395, 1890; xxviii, p. 210, 1891
[3] W. B. Hardy, *Journ. of Physiol.*, xxxiii, p. 251, 1905.

TABLE XVI.

(plus 1 c.c. of .05 per cent. trypsin)

C.C. of $\frac{N}{25}$ $Ca(OH)_2$	K (without NaCl)	K_1 (plus $\frac{N}{20}$ NaCl)	K_1 when K = 1
0	49	79	1.61
3	75.5	88	1.17
6	45	56	1.24
9	42	49	1.17

It must be remembered that since calcium caseinate is hydrolytically dissociated [1] and moreover probably consists, in solution, of a number of amphoteric bodies in equilibrium (ampho-salts, ampholates) [2] the figures in the first columns of the above tables only represent the nominal and not the actual excess of alkali. Nevertheless it is evident that *the acceleration of the hydrolysis produced by Sodium Chloride falls off as the alkalinity is increased.*

(g) *The Influence of the Concentration of Sodium Chloride upon the Acceleration which it Produces in the Hydrolysis of Casein by Trypsin.*

Experiment 1.—One hundred and fifty c.c. of an approximately $\frac{N}{1000}$ $Ca(OH)_2$ solution "saturated" with casein was placed in each flask and 0, 5, 10, 15, 20, 35, and 45 c.c. of a normal solution of sodium chloride added and each digest made up to 200 c.c.; no trypsin was added. The initial mass of casein in each digest was determined in the usual way and found to be 165 milligrams. Digestion (autohydrolysis) was carried on at 37° C. for three hours. The following were the results obtained.

TABLE XVII.

Concentration NaCl	K
0	18
0.025N	24
0.05 N	34.5
0.075N	31
0.1 N	48.5
0.175N	57
0.225N	31

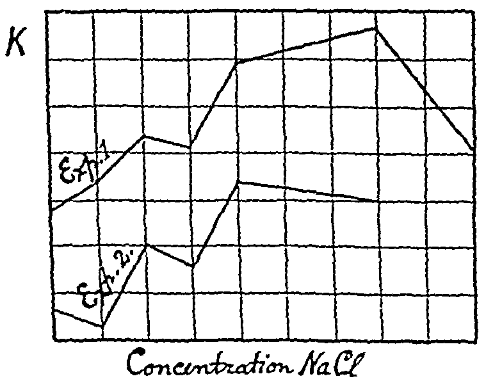
1 W. A. Osborne, *Journ. of Physiol.*, xxvii, p. 398, 1901.
2 Robertson, *Journ. of Physical Chem.*, x, p. 524, 1906.

Experiment 2.—The experiment was repeated in the same manner. The initial mass of casein in the digests was 149 milligrams. Digestion was carried on at 35.5° C. for three hours. The following were the results obtained.

TABLE XVIII.

Concentration NaCl	K
0	
0.025N	6
0.05 N	20
0.075N	16
0.1 N	34
0.175N	30

In the accompanying figure these results are plotted, the abcissæ being concentrations of sodium chloride and the ordinates the values for the velocity constants.



It cannot fail to be noted that the curves thus obtained bear a strong resemblance to those given by Oswald[1] repr- esenting the influence of the concentration of sodium chloride solution upon the amount of water taken up from it by gelatin plates. The theoretical significance of this resemblance will be discussed later.

(*h*) *The Influence of the Concentration of the Ferment upon the Acceleration of the Hydrolysis of Casein by Trypsin which is Produced by the Addition of Sodium Chloride.*

Two hundred c.c. of an approximately $\frac{N}{100}$ calcium hydrate

[1] Wolfgang Ostwald, *Arch. f. d. ges. Physiol.*, cxi, p. 518, 1906.

solution "saturated" with casein was placed in each flask and the flasks divided into two sets; to the one set were added 0, 1, 2, and 3 c.c. of a .05 per cent. solution of trypsin, while to the other set were added the same amounts of trypsin plus 10 c.c. of a normal sodium chloride solution. The initial amount of casein in each digest was 137 milligrams. Digestion was carried out at 36° C. for three hours. The following table embodies the results obtained.

TABLE XIX

C.C. of Ferment Solution.	K (without NaCl).	K_1 (with NaCl).	K_1 when K=1.
0	30	62	2.06
1	53	97	1.83
2	112	152	1.36
3	135	198	1.47

It will be seen that, in general, the acceleration produced by a given concentration of sodium chloride is less, relatively, the greater the concentration of the ferment.

(i) *The Changes in Reaction During Digestion.*

Gillespie [1] observed that as peptic digestion proceeded the amount of "free HCl" in the digest decreased. Tayl [2] has observed a diminution in the alkalinity of a tryptic digest of protamine sulphate as digestion proceeds. Greenwood and Saunders [3] have detected changes in the reactions to indicators as intracellular digestion proceeds.

Two hundred c.c. of a $\frac{N}{500}$ solution of calcium hydrate "saturated" with casein was placed in an Erlenmeyer flask and rendered just alkaline to phenolphthalein by the addition, drop by drop, of a solution of $\frac{N}{25}$ calcium hydroxide so that on addition of a few drops of phenolphthalein solution the digest took on a distinct pink hue. After digestion for two hours at 25–30° C. the pink color had disappeared, after an additional hour and a

[1] Gillespie, *Journ. of Anat. and Physiol.*, xxvii, p. 195, 1893.
[2] A. E. Taylor, *Univ. of Calif. Pub., Pathol.*, i, p. 7, 1904.
[3] Greenwood and Saunders, *Journ. of Physiol.*, xvi, p. 441, 1894.

half at 30–35° C. the color had completely gone, after two hours more at 35–40° C. the reactions to various indicators were tested and found to be as follows:

Neutral red	Red
Congo red	Rose
Tropæolin 000	Orange
Cyanin	Faint pale blue
Phenolphthalein	Colorless
Litmus	Blue-violet

This experiment was repeated a number of times both with and without trypsin; similar results were always obtained. The carbon dioxide of the air was, of course, excluded by tightly fitting rubber stoppers. Two hundred c.c. of a $\frac{N}{800}$ calcium hydroxide solution "saturated" with casein was placed in an Erlenmeyer flask and paranitrophenol added; $\frac{N}{40}$ hydrochloric acid was then run in drop by drop until the color, due to the paranitrophenol, had just completely disappeared. The casein was now partly precipitated and, possibly, owing to this the change in reaction took place very slowly at 38° C., but after twelve hours the characteristic greenish-yellow color of para nitrophenol in neutral or alkaline solution could be distinctly perceived. In twenty-four hours the color could not be distinguished from that of a neutral solution, although much of the casein still remained precipitated.

Hence both alkaline and acid digests of casein tend towards the neutral point as digestion proceeds. Neutral digests do not alter appreciably in reaction during digestion. The theoretical significance of these facts will be considered in the theoretical part of the paper.

IV. THEORETICAL.

In a recent paper[1] I have put forward the suggestion that the proteins may be regarded as associating amphoteric electrolytes (or, to use the term which I there suggested, associating ampho-

[1] Robertson, *Journ. of Physical Chem.*, x, p. 524, 1906.

lytes). The researches of Mann,[1] Billitzer,[2] Freundlich,[3] Laqueur,[4] Hardy,[5] and others have shown that proteins in solution act as electrolytes; their salts, in solution, undergo electrolytic[6] and hydrolytic[7] dissociation much as other salts do; while the fact that proteins are amphoteric in nature has been pointed out by Strecker,[8] Bredig,[9] Winkelblech,[10] Loeb,[11] Walker,[12] Hardy,[13] and Mann.[14]

The "association" in protein systems, that is, the formation of polymeric complexes, "pseudo-ions," etc., under various conditions has been discussed, among others, by Hardy.[15] Assuming, therefore, that proteins are to be regarded as associating ampholytes, I suggested, in the paper to which I have referred, that the compounds which have hitherto been termed "ion-proteids," as also the "molecular compounds" between proteins and acids, bases, or salts, might be regarded as ampho-

1 Gustav Mann, *Physiological Histology*, Oxford, 1902, p. 45; *Chemistry of the Proteids*, 1906, pp. 259, 268.

2 J. Billitzer, *Ann. d. Physik.*, cccxvi, pp. 902, 937, 1903.

3 H. Freundlich, *Zeitschr. f. physik. Chem.*, xliv, p. 129, 1903.

4 Laqueur, Inaug. Diss., Breslau, 1905; *Biochem. Centralbl.*, iii, p. 670.

5 W. B. Hardy, *Journ. of Physiol.*, xxxiii, p. 251, 1905.

6 In regard to the evidence that protein salts are electrolytically dissociated, see the authors just referred to, also Huiskamp, *Zeitschr. f. physiol. Chem.*, xxxiv, p. 32, 1901, to whose work I regret that I omitted to refer in my previous paper (*loc. cit.*).

7 In regard to the hydrolytic dissociation of proteins see: Erb, *Zeitschr. f. Biol.*, xli, p. 309, 1901. Sjöqvist, *Skand. Arch. f. Physiol.*, v, p. 277. 1894. Arrhenius and Ley, *Zeitschr. f. physikal. Chem.*, xxx, p. 193, 1899. Bugarsky and Liebermann, *Arch. f. d. ges. Physiol.*, lxxii, p. 51, 1898. Spiro and Pemsel, *Zeitschr. f. physiol. Chem.*, xxvi, p. 233, 1898. W. A. Osborne, *Journ. of Physiol.*, xxvii, p. 398, 1901. Laqueur, *loc. cit.* W. B. Hardy, *Journ. of Physiol.*, xxxiii, p. 251, 1905.

8 Strecker, *Ann. d. Chem.*, cxlviii, p. 87, 1886.

9 Bredig, *Zeitschr. f. Electrochem.*, 1899.

10 Winkelblech, "Ueber amphotere Elektrolyte und innere Salze," Inaug. Diss., Leipzig, 1901; quoted after Mann, *Chemistry of the Proteids* 1906, p. 208; *Zeitschr. f. physikal. Chem.*, xxxvi, p. 546, 1901.

11 J. Loeb, *Univ. of Calif. Pub., Physiol.*, 1, p. 149, 1904.

12 Walker, *ibid.*, xlix, p. 82, 1904.

13 W. B. Hardy, *loc. cit.*

14 Gustav Mann, *Chemistry of the Proteids*, 1906, pp. 145 and 208.

15 W. B. Hardy, *loc. cit.*

salts or di-salts of the ampholyte (types Na + XOH, HX + Cl, NaX + Cl, Na + XCl), while the "non-dissociable" ion-proteid compounds might be regarded as "acid" or "basic" ampho-salts[1] or as di-salts (types $H^+ + XCl'$, $NaX^+ + OH'$, $NaX^+ + Cl'$, $Na^+ + XCl'$).

Regarded from this point of view it will be seen that a solution of casein in lime-water cannot be regarded as a solution of a single substance, casein, but rather as a mixture of polymeric modifications and compounds of casein. W. A. Osborne,[2] Sackur[3] and Laqueur[4] have pointed out this complexity in solutions of casein, while Hardy[5] has drawn attention to similar phenomena in globulin solutions.

The evidence for the existence of definite salts of casein containing definite proportions of bases rests upon the facts that, at definite hydrion concentrations (as determined by the neutral points of given indicators), a given quantity of casein neutralizes equivalent molecular proportions of different bases and that the alcohol precipitate contains, also, equivalent molecular percentages of the base. This constitutes no proof that these compounds are simple salts of the bases in the ordinary sense of the word. The above facts are equally easily explained by assuming the existence of a mixture, as outlined above, for, as I have previously pointed out,[6] at a given hydrion concentration the proportion of dissociated basic ampholates to dissociated acid ampholates is constant, and hence it is not surprising that, at a given hydrion concentration, a protein of Class I (see paper to which reference has just been made) should neutralize equivalent molecular proportions of different bases, for the proportion of the active mass of protein acid to the active mass of protein base would be constant at any given hydrion concentration. Estimates of the molecular weight of

[1] J. Loeb, *Dynamics of Living Matter*, Columbia Univ. Press, 1906, p. also 72.

[2] W. A. Osborne, *Journ. of Physiol.*, xxvii, p. 398, 1901.

[3] Sackur, *Zeitschr. f. physik. Chem.*, ix, p. 487, 1892.

[4] Laqueur, Inaug. Diss., Ereslau, 1905; *Biochem. Centralbl.*, iii, p. 670.

[5] W. B. Hardy, *Journ. of Physiol.*, xxxiii, p. 251, 1905.

[6] Robertson, *loc. cit.*

casein, therefore, based on the determinations of the percentage of calcium hydrate neutralized by casein at a given hydrion concentration, are premature until we are certain that we are dealing with a single substance.

The question now arises, which of the constituents of the mixture is attacked by trypsin? Since casein neutralizes bases in equivalent molecular proportion, if the sodium ampho-salt has a formula which may be written NaXOH, then the calcium salt should have the formula, $Ca(XOH)_2$. If the trypsin reacted with the ampho-salts, therefore, we should expect, if one molecule of trypsin reacted with one molecule of the sodium ampho-salt, two should react with one of the calcium salt—so that, if the velocity of the hydrolysis of sodium caseinate were directly proportional to the mass of the trypsin, the velocity of hydrolysis of the calcium caseinate should be proportional to the *square* of the ferment concentration. As we have seen, however, this is not the case. In both instances the velocity is directly proportional to the ferment mass within certain limits of substrate concentration, so that I am led to conclude that the trypsin reacts, at moderate substrate concentration, only with *the anions and cations of the dissociated acid and basic ampholates* (*types* $H^+ + XOH'$, $HX^+ + OH'$) *set free by hydrolytic or electrolytic dissociation.*

At a higher substrate concentration [$4\frac{N}{100}$ $Ca(OH)_2$ "saturated" with casein] the ampho-salts apparently enter into the reaction and we have the sum of two effects, one the influence of the trypsin upon the hydrolysis of the ampholates, the other the influence upon the ampho-salts; hence, in the case of calcium caseinate at high substrate concentration, the velocity constant of hydrolysis varies as $af + bf^2$ where a and b are constants and f is the ferment concentration, the linear factor representing the influence of the ferment upon the hydrolysis of the ampholates, and the quadratic factor representing the influence upon the hydrolysis of the calcium ampho-salt, in which, as I have pointed out, *two* molecules of the ferment are in all probability involved. If this explanation were correct we should expect that the relation between ferment concentration and velocity of hydrolysis of caseinates of monacid bases should remain approximately linear

for all substrate concentrations, since whether the ampho-salts or ampholates are involved, only one molecule of ferment reacts with each molecule of substrate; I have made a few observations on the influence of ferment concentration upon the hydrolysis of concentrate dsodium caseinate ($\frac{N}{400}$ NaOH saturated with casein) and have obtained a linear relation with no trace of a quadratic factor. Bayliss, using about 5 per cent. sodium and ammonium caseinates—corresponding to about $\frac{N}{25}$ alkali "saturated" with casein,—found a linear relation between ferment concentration and the initial velocity of hydrolysis; the relation is therefore linear for caseinates of monacid bases over a very wide range of substrate concentration and at nearly twenty times the substrate concentration at which the quadratic factor first appears in the equation representing the relation between the velocity of hydrolysis of *calcium* caseinate and the ferment concentration.

Since, in casein, the undissociated ampholates and association-salts (types HXOH, XX) are practically insoluble, an increase in the solubility of casein probably involves an increase in dissociation, since, as I have elsewhere shown,[1] in an associating ampholyte system, dissociation increases as association (and the consequent formation of undissociated ampholates and association-salts) diminishes. Any salt, therefore, which increases the solubility of casein in the solution to which it is added must, for that reason, increase the concentration of dissociated acid and basic ampholate anions and cations in such a solution containing a caseinate and thus, by increasing the mass of the substrate actually attacked by the trypsin, must increase the velocity of hydrolysis. Salts which diminish the solubility of casein would have the reverse effect. In confirmation of this idea I mayallude to the fact that, as we have seen, those salts which increase most the solubility of casein in calcium hydrate also diminish to the greatest extent the opalescence of solutions of calcium caseinate to which they are added, indicating a diminution in the number of pseudo-ions and association-salts present in the solution. We should thus expect to find a close correspondence between the

[1] Robertson, *loc. cit.*

increase in solvent power for casein conferred by salts on calcium hydrate and the acceleration of the hydrolysis of calcium caseinate by trypsin which they produce. The close agreement between the facts and the hypothesis cannot fail to be noted on comparing Tables IV and XIV. That the correspondence is not absolutely complete is readily understood when we recollect that the dissociation of the ferment may also be affected by the salt. Nasse[1] and Chittenden and Cummins[2] concluded that the action of salts upon ferment reactions was specific for each individual ferment. In the face of such contradictory results as had already been obtained in investigations upon the influence of salts on tryptic digestion it is difficult to understand how these authors felt themselves justified in reaching this conclusion. Chittenden and Cummins used fibrin as substrate in neutral solution and found that most salts retarded the digestion by trypsin. I have used calcium caseinate as substrate in neutral solution and I find that nearly all salts accelerate the digestion—in the same way an examination of the literature will show that hardly a single statement has been made by any observer regarding the influence of dissolved substances upon tryptic digestion which has not been contradicted by some other observer. Divergences such as these are not to be attributed to experimental errors. On the basis of the hypothesis that the influence of salts upon tryptic digestion is to be attributed in the main to alterations in the dissociation of the substrate, the widely differing results obtained when different substrates are used become explicable.

It might possibly be argued that on adding a salt to a solution of a caseinate the salt may partially replace some of the alkali in the compound and that the alkali thus set free might be responsible for the acceleration. That this is not the case is seen on referring to Table VI, the addition of tenth-normal solutions of salts to solutions of ᴺ $Ca(OH)_2$ "saturated" with casein, with the exceptions of potassium acetate and potassium cyanide, only raising the hydroxyl concentration to an alkalinity which,

[1] Nasse, *Arch. f. d. ges. Physiol.*, xi, p. 157, 1875.
[2] Chittenden and Cummins, *Trans. Connecticut Acad.*, vii, p. 108.

as other experiments show, produces scarcely any appreciable acceleration.

The dissociation of the ampholates having reached a maximum. the addition of a salt to the solution would no longer produce any acceleration of the digestion, hence, as we have seen, after the addition of an excess of alkali to a solution of neutral calcium caseinate (which must, obviously, increase the dissociation and active mass of the acid ampholates) the addition of sodium chloride produces less relative acceleration of the hydrolysis.

In my paper on the conditions of equilibrium of an associating ampholyte, to which I have referred, I have pointed out that the degree of "swelling" which a protein attains in a given solution in all probability runs parallel with its solubility in that solution. Now the velocity of the hydrolysis of a protein by trypsin, on the basis of the hypothesis which I have put forward, is proportional, other factors being constant, to the solubility of the substrate in the medium—hence the influence of concentration of a salt upon the acceleration of hydrolysis produced by it should be similar to the influence of concentration of a salt upon the swelling of a protein in its solution. The close similarity between the curve representing the relation of concentration to acceleration of tryptic digestion (see figure, page 367), and that representing the influence of concentration on the swelling of gelatin as found by Wolfgang Ostwald, was therefore to be expected and admits of a ready explanation, namely, that increase in swelling and increase in velocity of hydrolysis due to the addition of a salt are in the main due to the same thing, that is, to the increase in the dissociation of the acid and basic ampholates and consequent diminution in association.

The solvent powers of salts and alkalies for casein are not, in general, additive; as I have pointed out elsewhere this is to be expected in the case of a protein which neutralizes bases or acids in *equivalent molecular* proportions.[1]

In a recent paper Bayliss[2] has suggested that enzyme action, and, in particular, tryptic digestion of proteins, may be, in part at least, "adsorption phenomena." Bayliss himself, however,

[1] Robertson, *loc. cit.*

[2] W. M. Bayliss, *Biochem. Journ.*, i, p. 175, 1906.

finds that the temperature coefficient for what he considers a typical case of adsorption, the abstraction of Congo red from its solution by filter paper, is very low, namely 1.36, while the temperature coefficient for the digestion of casein by trypsin is unusually high even for a chemical reaction, being 3.3 between 30° and 40° C. and estimated at 12 for 10° between 0° and 30° C.[1] These abnormally high temperature coefficients are not at all what would be expected in what are generally understood as adsorption phenomena, but they are precisely what are found for reactions involving amphoteric electrolytes.[2] The abnormally high temperature coefficients obtained for reactions involving ampholytes have been attributed to the very rapid increase in dissociation of amphoteric electrolytes as the temperature rises.[3]

The influence of salts upon the hydrolysis of calcium caseinate by trypsin is, it is true, that which might be expected if the acceleration in hydrolysis were due, in the first place, to the "adsorption" of the ferment by the substrate, for since casein and trypsin are both predominantly acid in character, that is, are "negative" colloids, adsorption of the one by the other would be accelerated by neutral salts.[4] In view of the above considerations, of the widely differing effects of salts upon hydrolysis when different substrates are used, and of the fact that this influence of salts upon the hydrolysis of calcium caseinate was to be expected upon other grounds, I do not think that this fact affords a very strong argument for adsorption as opposed to reaction by chemical equivalents.

As Hardy[5] has pointed out "recent work on complex ions has extended the possibility of reaction by chemical equivalents to a point where it seems to be indistinguishable from adsorption combination," and a glance at the formulæ for the interaction

[1] Bayliss, *Arch. des sciences biol.*, xi, Suppl., p. 261, 1904. Reprinted in the Collected Papers of the Physiological Laboratory, University College, London, vol. xiii.

[2] Hantzsch, *Ber. deutsch. chem. Gesellsch.*, xxxii, p. 3066, 1899.

[3] Harold Lundén, *Zeitschr. f. physik. Chem.*, liv, p. 532, 1906.

[4] Bayliss, *Biochem. Journ.*, i, p. 175, 1906.

[5] W. B. Hardy, *Journ. of Physiol.*, xxxi, p. 302, 1905.

of two associating ampholytes, which are given in my paper on the equilibrium of an associating ampholyte, will serve to reveal the fact that in this direction also the possibilities of reaction by chemical equivalents can be extended to cover such phenomena.

If "adsorption combination" be defined as "combination by chemical equivalents in systems containing a large and indefinable number of reacting units," there can, of course, be no doubt as to its actual existence, but at the same time the term becomes unnecessary. If, on the other hand, "adsorption combination" be defined as a purely physical phenomenon, an alteration in the distribution of a substance due to changes in surface energy, such as the retention of potassium permanganate by quartz sand on the filtration of a solution through it, its existence, again, cannot be questioned, but, again, the term is unnecessary and its introduction as a prime factor in reactions unquestionably accompanied by definite chemical changes (such as the hydrolysis of proteins by trypsin) would appear of doubtful utility.

If, however, "adsorption combination" be defined as an expression of "mechanical affinity," [1] as a semi-chemical phenomenon distinct from reaction by chemical equivalents, while the possibilities of reaction by chemical equivalents, opened out by the modern conceptions of valency, complex ions, association compounds, ampholytes, etc., are so far from being exhausted, it would appear premature to state definitely that reactions taking place in admittedly complex and undefined chemical systems are not essentially chemical in character. There are indications that the phenomenon of chemical reaction by equivalents is a fundamental one, based on the structure of the atom and of the molecule, and until the possibilities opened up by the recent chemical conceptions to which I have alluded have been definitely tried and found wanting, reference to "adsorption combination" or "mechanical affinity" in protein systems appears likely to act rather as a deterrent from, than as a stimulus to, research. That no definite stochiometrical relations should be found to exist between two complex systems of chemical entities (such as two associating ampholytes, etc.), each of the systems being taken as a unit, is not surprising and it does not prove that

[1] Ostwald, *Lehrbuch d. allgem. Chem.*, 2te Aufl., i, p. 1084, *et seq.*

definite stochiometrical relations do not subsist between the individual constituents of the systems. The recognition of the enormous importance of surface and "adhesion" phenomena in protein systems does not, to my mind, imply the denial in any specific case of the law of chemical reaction by equivalents, *and the introduction of these phenomena* as explanatory in the field of definite chemical changes must be accepted with great caution unless as a part of a general chemical theory.

The contention of Dauwe [1] that the action between substrate and enzyme cannot be chemical because the enzyme, after having been taken up by such substances as coagulated white of egg, can be extracted again by an appropriate solvent, fails in the light of the behavior of such acknowledged chemical compounds as mercuric sulphate and cupric chloride, the sulphuric acid or hydrochloric acid radicles of which can be completely removed by trituration with water.[2]

The hypothesis advanced by Herzog,[3] following Nernst, that the reaction between trypsin and a protein substrate takes place at the interface of the colloid substrate and the medium at a practically infinite velocity on account of the high chemical potential at the surface, so that the apparent velocity of the reaction is practically dependent upon the velocity of diffusion across the interface, rests upon a different basis from the hypotheses we have hitherto been considering, inasmuch as it falls within the known possibilities of reaction by chemical equivalents. In the present instance, however, casein being used as substrate, the temperature coefficient would appear to indicate that the velocity measured is a chemical velocity and not a diffusion velocity; with other substrates, however, the contrary may possibly be the case.[4]

[1] Dauwe, *Beitr. z. chem. Physiol. u. Path.*, vi, p. 426, 1905.

[2] See, for example, Lescœur, *Ann. de chim. et de phys.*, 7 sér., ii, p. 97, 1894. Haywood, *Journ. of Physical Chem.*, i, p. 411, 1897.

[3] Herzog, *Zeitschr. f. physiol. Chem.*, xli, p. 416, 1904; *ibid.*, xlviii, p. 365, 1906.

[4] For instance A. E. Taylor (this Journal, ii, p. 87, 1906) has recently found that in the hydrolysis of an ester of low molecular weight by lipase the temperature coefficient is that of a chemical reaction, while when a colloidal ester of very high molecular weight is used as substrate the temperature coefficient is that of diffusion velocity.

It is generally acknowledged that in protein hydrolysis by ferments the ferment combines in some way with the substrate.[1] Assuming that one molecule of trypsin combines with one molecule of protein, the compound subsequently splitting up, setting free the primary products of digestion and regenerating the ferment, the most obvious relation to be expected between the time of hydrolysis and the amount of substrate hydrolyzed is that which would obtain in a bimolecular reaction, one of the reacting substances (in this case the ferment) remaining of constant concentration. This would give a relation $\log \frac{a}{a-z} = kft$, where a is the initial substrate concentration, z is the amount hydrolyzed, f is the ferment concentration, t is the time, and k is a constant, and this is the relation which, as we have seen, has been found experimentally. On closer analysis of the kinetics of such a reaction, however, this relation is not by any means so obvious as might be supposed. Suppose that at any moment the concentration of the ferment-substrate compound is x, while that of the products of hydrolysis is y, then we have at that moment, provided the retarding influence of the small concentration of the ferment-substrate compound can be neglected:—

$$\frac{dx}{dt} = k_1 f(a-x-y)$$

hence, if $$z = x + y$$

$$\frac{dz}{dt} = \frac{dx}{dt} + \frac{dy}{dt}$$

$$\therefore \quad \frac{dz}{dt} = k_1 f(a-z) + \frac{dy}{dt}$$

In order to recover the equation experimentally obtained, in general, $\frac{dy}{dt}$ must be negligible, that is, we must suppose that the second reaction proceeds so rapidly that the velocity which is measured is that of the *combination* between the ferment and the substrate, and the products of the second reaction may be

[1] See, for instance, Vernon, *Journ. of Physiol.*, xxxi, p. 346, 1904; V. Henri, *Compt. rend. de la soc. biol.*, lviii, p. 610; Bayliss, *Arch. des sci. biol.*, xi, Suppl., p. 261, 1904, reprinted in the Collected Papers of the Physiological Laboratory, University College, London, vol. xiii.

regarded, for experimental purposes, as being, at any given moment, in equilibrium with the ferment-substrate compound.

In the second stage of the reaction the ferment-substrate compound must break down, yielding ampholates of a lower order than the substrate and regenerating the ferment.[1] It is not necessary to suppose that the subsequent stages of the reaction take place at a velocity according with the monomolecular formula, nor, indeed, to consider the rate of change of y at all except in so far as it tends to modify the concentration of uncombined ferment. For the rate of change of the substrate being the only quantity measured in the experiments herein described, since the second stage of the reaction proceeds at a practically infinite velocity, any irregularities in the rate of change of the products of this reaction cannot alter the form of the expression for the rate of change of the substrate until the concentration of the products becomes so great that their depressant effect becomes measurable. Hence a monomolecular formula is more likely to be obtained for a reaction proceeding in multiple stages if, as in these experiments, the rate of change of the *substrate* is measured rather than the rate of appearance of the *products*.

The *alterations in reaction* of an alkaline or acid digest, as digestion proceeds, admit, I think, of a simple explanation upon the basis of the above conceptions. Suppose we have an alkaline digest to begin with, then the major part of the ampholates will be acid in character. Suppose now that a molecule of the substrate splits up, two ampholates of a lower order will be produced, and since for the consequent slight alteration in hydroxyl concentration the acid character of the ampholates will not be sensibly altered, the products will neutralize nearly twice as much of the alkali as the original molecule of the substrate. Obviously, the alkalinity of the solution will diminish, the more slowly as time proceeds, and will tend asymptotically towards the value at which the concentration of the acid ampholates equals

[1] Possibly, in this stage, a *product* of the ferment is formed and subsequently regenerates the ferment. Since the masses of the two forms of the ferment would always be proportional the equations for velocity, etc., would remain of the same form.

that of the basic ampholates, that is, towards the "iseloectric" point. This obviously corresponds with the facts observed.

V. CONCLUSIONS.

1. A suspension of casein in distilled water reddens litmus paper wherever the suspended particles touch it, but if this suspension be filtered the filtrate contains no detectable casein and is neutral to litmus. An explanation is suggested.

2. A volumetric method of estimating casein quantitatively is described and examples are given of the degree of accuracy attainable.

3. The maximum amount of casein which a given concentration of calcium hydrate will hold in solution is that with which it will combine to form neutral calcium caseinate, and the reaction of the resultant solution, as estimated by indicators, is from 10^{-6}N to 10^{-7}N.

4. No evidence was found of the existence of compounds of casein with bases containing a greater proportion of casein than the neutral caseinates.

5. The solubility of casein in a variety of salts has been estimated. Casein is most soluble in solutions of potassium cyanide, then follow the sodium and potassium salts of the fatty acids, while it is least soluble in solutions of the chlorides, sulphates, and nitrates of the alkalies.

6. The solubility of casein in solutions of calcium hydrate to which various salts are added has been estimated. The order in which the salts augment the solubility of casein in calcium hydrate solutions is similar to that in which solutions of the salts alone dissolve casein; there are divergences, however, and the solvent powers of calcium hydrate and the various salts, for casein, are not additive.

7. In equally concentrated solutions of casein the absence of opalescence runs parallel with the solubility of casein in the solutions.

8. On adding calcium chloride to a solution of neutral calcium caseinate the casein is precipitated, presumably owing to the diminution of dissociation of the caseinate through the addition of the common ion.

9. The reactions to indicators of solutions of neutral calcium caseinate to which varying quantities of alkali or acid have been added are described.

10. The reactions to indicators of solutions of neutral calcium caseinate to which various salts have been added are described.

11. Grübler's trypsin, in watery solution, has an acid reaction and acts like an acid in forming an insoluble compound with the color base safranin.

12. Neutral caseinates, in solution, undergo fairly rapid autohydrolysis, one third of the substrate being hydrolyzed in twelve hours at 38 ° C. The hydrolysis of the caseinates by trypsin, therefore, affords an excellent example of the action of an enzyme in accelerating an already existing reaction.

13. The velocity of the hydrolysis of calcium caseinate by trypsin at moderate substrate concentration [$\frac{N}{80}$ $Ca(OH)_2$ and lower concentrations "saturated" with casein] is directly proportional to the amount of trypsin present—at higher substrate concentrations [$\frac{N}{40}$ $Ca(OH)_2$ "saturated" with casein], if f is the concentration of the ferment, the velocity constant of the hydrolysis is equal to $af+bf^2$ where a and b are constants. The velocity of hydrolysis of neutral sodium caseinate appears to be directly proportional to the ferment concentration at all substrate concentrations. In explanation it is suggested that at low substrate concentration the trypsin only attacks the anions and cations of the dissociated acid and basic ampholates (types $H^+ + XOH'$, $HX^+ + OH'$) set free by hydrolytic and electrolytic dissociation, while at higher substrate concentration the ampho-salts [types $Na^+ + XOH'$, $NaX^+ + OH'$, $Ca^{++} + (XOH)'_2$, $CaX^{++} + OH'_2$] also enter into the reaction.

14. The relation between the amount of calcium caseinate hydrolyzed and the time of hydrolysis is, for the earlier stages of the reaction, what would be expected from the formula, $\log \frac{a}{a-x} = Kt$.

15. The influence of a variety of salts upon the velocity of the hydrolysis of calcium and sodium caseinates by trypsin is described. In general, the order in which salts augment the solubility of casein in calcium hydroxide and in which they diminish the opalescence of a solution of calcium caseinate is

also the order in which they increase the velocity of the hydrolysis.

16. The acceleration of the hydrolysis of calcium caseinate produced by sodium chloride falls off as the alkalinity is increased.

17. The acceleration of the hydrolysis of calcium caseinate produced by a given concentration of sodium chloride is less, relatively, the greater the concentration of the ferment.

18. The influence of the concentration of sodium chloride upon the velocity of the hydrolysis of calcium caseinate is similar to its influence upon the degree of swelling of gelatin plates in sodium chloride solutions.

19. The hydrion concentration in alkaline or acid digests of calcium caseinate tends towards neutrality as digestion proceeds. The reaction of neutral solutions of calcium caseinate does not appreciably change during digestion.

20. In the theoretical part of the paper the above facts are shown to be in harmony with the view that proteins may be considered as amphoteric electrolytes which undergo association in solution, while the salts of the proteins, or the "ion-proteids," can be regarded as ampho-salts or disalts (types $Na^+ + XOH'$, $HX^+ + Cl'$, $Na^+ + XCl'$, $NaX^+ + Cl'$). The reaction between a proteoclastic enzyme and a protein is regarded as a reaction between two such associating ampholytes.

21. Reasons are advanced for regarding the action between the protein and the ferment as taking place in successive groups of two stages each, the second reaction being supposed to proceed at a very much greater velocity than the first.

Finally, I wish to express my indebtedness to Dr. A. E. Taylor for valuable hints regarding technique and manipulation and for the loan of certain apparatus, and to Dr. Loeb for his unremitting interest in the work and the manner in which he placed the resources of the laboratory at my disposal.

THE ACTION OF CERTAIN VEGETABLE CATHARTICS ON THE ISOLATED CENTRE OF A JELLY-FISH (*POLYORCHIS*).

By JOHN BRUCE MACCALLUM.

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(Received for publication, March 1, 1906.)

Several years ago Dr. Loeb[1] described the effect of various salt solutions on the isolated centre of a jelly-fish of the Atlantic Coast (*Gonionemus*). He found that the centre of the jelly-fish, which, as Romanes had observed, did not beat in sea-water when separated from the margin, might be caused to beat rhthmically by placing it in pure sodium chloride solution. These contractions could be inhibited by the addition of calcium or magnesium chloride. Further, the isolated centre could be made to beat in sea water if there were added, in excess, any salt which precipitates calcium. The salts which were found by Loeb to cause these rhythmical contractions include those commonly known as saline purgatives. This was a continuation of work done on the skeletal muscles of the frog, in which it was shown[2] that rhythmical twitchings could be produced by a group of salts including the saline purgatives and could be inhibited by calcium or magnesium. This suggested experiments which were carried out on the intestines of rabbits, in which it was shown[3] that the saline purgatives produce rhythmical and peristaltic movements of the intestines not only when introduced intravenously and subcutaneously, but also when applied locally to the peritoneal surface of the intestines. Further, loops of the intestines isolated from the body show active rhythmical contractions when placed in solutions of these

[1] Loeb, J., *Amer. Journ. of Physiol.*, iii, p. 383, 1900.

[2] Loeb, J., *Festschrift für Professor Fick*, Braunschweig, 1899.

[3] MacCallum, J.B., *Amer. Journ. of Physiol.*, x, pp. 101 and 259, 1903–04.

salts. It was also shown[1] that the dried extract of cascara sagrada, when dissolved in a diluted solution of sodium bicarbonate, is capable of causing active peristaltic movements when given intravenously or subcutaneously or applied locally to the peritoneal surface of the intestines. The experiments of Loeb on the centre of *Gonionemus* and his later experiments on *Polyorchis*, a jelly-fish found in the Pacific, suggested the possibility of testing the action of many of the vegetable purgatives on the isolated centre of this animal. This organism is of especial interest for such experiments because of the ease with which the contractions can be watched, and on account of the fact that the centre can be almost completely isolated from the nervous system, which is present in a ring about the margin. The following is a report of a number of experiments made with *Polyorchis* to test the action of various vegetable purgatives.

The methods used were practically those employed by Dr. Loeb in his experiments with salts. Mixtures were made of various concentrations of the purgative solutions with sea water. These mixtures were placed in finger bowls on a black surface. The jelly-fishes were cut in two with scissors just above the row of sense organs so that the entire nervous ring was separated from the centre of the animal. This centre, which under no circumstances beats in pure sea water, was then placed in the mixture of sea water and purgative. In each case the optimal and minimal concentrations for the production of contractions was observed.

Cascara Sagrada. The dried extract of this substance as it is ordinarily obtained is practically insoluble in water. When mixed with distilled water a dirty yellow suspension results with apparently little, if any, of the powder in solution. The mixture has quite a strong acid reaction (litmus). The addition of a small quantity of sodium bicarbonate causes the suspended powder to be almost immediately dissolved with the production of a clear dark brown solution. The addition of a drop or two of an acid (*e.g.* sulphuric acid) precipitates the cascara again. It seems that the extract is insoluble in water on account of the

[1] MacCallum, J. B., *University of California Publications, Physiology*, i, p. 115, 1904.

acid it contains. When this is neutralized the cascara goes into solution.

Solutions for experiment were therefore made by dissolving the dried extract in dilute sodium bicarbonate solution. It is possible to readily dissolve 0.5 gram of extract in 25 c.c. of $\frac{M}{24}$ sodium bicarbonate. Somewhat less concentrated solutions than this were used.

With a solution of 0.125 gram of cascara in 50 c.c. of $\frac{M}{24}$ sodium bicarbonate, mixtures were made as follows: (1) 25 c.c. of sea water and 1 c.c. of cascara solution; (2) 25 c.c. of sea water and 2 c.c. of cascara solution; (3) 25 c.c. of sea water and 3 c.c. of cascara solution; (4) 25 c.c. of sea water and 5 c.c. of cascara solution. In Mixtures 1 and 2 no contractions were produced. In Mixture 3 active contractions appeared but with some inconstancy. This concentration did not always seem sufficient to produce the movements. In Mixture 4 active movements began at once and continued regularly for five minutes, sometimes longer. These were quite constant, and of a regular rhythm.

With a solution of 0.25 gram of cascara in 50 c.c. of $\frac{M}{24}$ sodium bicarbonate, mixtures were made as follows: (1) 25 c.c. of sea water and 1 c.c. of cascara solution; (2) 25 c.c. of sea water and 2 c.c. of cascara solution; (3) 25 c.c. of sea water and 5 c.c. of cascara solution. In Mixture 1 there developed only occasional contractions and no rhythmical beating was produced. In Mixture 2 strong regular beats began at once and continued for ten minutes or more. In Mixture 3 very powerful beats began at once but lasted only a few minutes.

With cascara, therefore, it seems that the optimal concentration is about that of Mixture 2 in the last series, while the minimal concentration is between Mixtures 1 and 2.

These experiments are complicated by the presence of sodium bicarbonate in the solution. Control experiments were made with mixtures made up of sea water and $\frac{M}{24}$ sodium carbonate solution in the same proportion as the above mixture of sea water and cascara solution. With all of these mixtures of sea water and sodium bicarbonate no contractions developed. Indeed, Professor Loeb has told me that he has found that the contractions of the isolated centre are inhibited by an

alkali. The presence of sodium bicarbonate, therefore, in the above experiments would tend to inhibit the action of the cascara. This applies also to those substances spoken of below which it was necessary to dissolve in sodium bicarbonate.

Rhubarb. The powdered dry extract of rhubarb like that of cascara sagrada is practically insoluble in water. It may be brought into solution by the same process as that described above for cascara. They are soluble to about the same extent in $\frac{M}{24}$ sodium bicarbonate. A solution was made up of 0.125 gram of dry extract of rhubarb in 50 c.c. of $\frac{M}{24}$ sodium bicarbonate. A very dark brown solution resulted. The following mixtures were then made and tested: (1) 25 c.c. of sea water and 0.1 c.c. of rhubarb solution; (2) 25 c.c. of sea water and 0.25 c.c. of rhubarb solution; (3) 25 c.c. of sea water and 0.5 c.c. of rhubarb solution; (4) 25 c.c. of sea water and 1 c.c. of rhubarb; (5) 25 c.c. of sea water and 2 c.c. of rhubarb; (6) 25 c.c. of sea water and 3 c.c. of rhubarb; (7) 25 c.c. of sea water and 5 c.c. of rhubarb.

In Mixture 1 no contractions whatever developed and the centre of the jelly-fish was quite non-irritable to slight mechanical stimulations, such as shaking or touching with a glass rod.

In Mixture 2 occasional feeble contractions usually appeared, but no regular beating of the centre was produced. The centre was not irritable to the mechanical disturbances mentioned above.

In Mixture 3 the isolated centre began to beat as soon as it was dropped into the fluid. These were strong regular beats averaging at first about 52 to the minute. They gradually became slower and after having gone on vigorously for ten to fifteen minutes they ceased. When, however, the finger-bowl containing it was gently shaken the centre renewed its beating and gave half a dozen or more fairly strong contractions. Touching it with a glass rod or the point of a forceps had a similar effect. In other words, it remained irritable to such stimulations after the contractions produced by the drug had ceased.

In Mixture 4 the beating of the centre developed and continued in the same way as in Mixture 3. Some of the centres placed in this solution beat for more than fifteen minutes and

remained quite irritable for a time after the contractions had ceased.

It may be mentioned that usually only one and never more than two centres were placed in the same finger-bowl containing only 25 c.c. fluid. This was to make it certain that the volume of fluid would be relatively great as compared with the volume of the centre.

In Mixtures 5, 6, and 7 strong contractions began as soon as the centre was placed in the mixture. They, however, were more vigorous than lasting in character, and after five to six minutes they nearly always ceased. In stronger solutions they stopped still sooner.

The optimal concentration of rhubarb for the production of rhythmical contractions in the isolated centre of *Polyorchis* seems to be between that of Mixtures 3 and 4. The minimal concentration is about that of Mixture 2.

Aloin. This substance is quite readily dissolved in water. A solution was made of 0.1 gram of powdered aloin in 25 c.c. of distilled water. Mixtures were then made as follows: (1) 25 c.c. of sea water and 1 c.c. of aloin solution; (2) 25 c.c. of sea water and 3 c.c. of aloin solution; (3) 25 c.c. of sea water and 4 c.c. of aloin; (4) 25 c.c. of sea water and 5 c.c. of aloin; (5) 25 c.c. of sea water and 6 c.c. of aloin solution.

In Mixtures 1 and 2 no contractions developed in the isolated centre. In Mixture 3 occasional feeble contractions occurred. These were irregular and soon stopped. The centre was not irritable to mechanical stimulations in this solution.

In Mixture 4 regular beating of the centre began at once and continued for ten minutes. These contractions were strong and regular. The centre was irritable when shaken or touched for some time after the contractions produced by the aloin had ceased.

In Mixture 5 the beating of the centre began as soon as it was immersed. The contractions were strong and regular and somewhat more rapid than in Mixture 4. The centre continued to beat for fifteen to twenty minutes, the contractions gradually becoming slower until they ceased. After this the centre was still irritable and contracted on being touched. This mixture seemed to be the optimal one in the case of aloin, while the minimal concentration is about that of Mixture 3.

Podophyllin. This substance dissolves only to a very slight extent in water. In $\frac{M}{24}$ sodium bicarbonate a considerable amount goes into solution. A solution was made by shaking 0.5 gram of podophyllin in 50 c.c. of $\frac{M}{24}$ sodium bicarbonate. This made a brown mixture in which much was undissolved. The filtrate from this was a clear brownish fluid. This was used for the experiment, though the concentration was not known. Mixtures were then made of 1, 2, 5, and 6 c.c. of this solution in 25 c.c. of sea water as before. In the first two mixtures no contractions developed in the isolated centre of the jelly-fish. In the last two mixtures very active beating of the centre began at once and continued for three to five minutes. These contractions were at first quite regular but became jerky and uneven, as though different parts of the centre contracted at different times. The minimal concentration lies between that of the second and third mixtures.

Colocynth. The action of colocynth proved to be uncertain. It is fairly soluble in water, 0.5 gram of colocynth being dissolved in 25 c.c. of distilled water. No contractions were obtained with mixtures less concentrated than 25 c.c. of sea water and 10 c.c. of this colocynth solution. In this mixture regular contractions were obtained in a number of cases, but did not appear in other instances. Still stronger solutions were no more certain in their effect.

It is evident from these experiments that the vegetable purgatives stimulate the isolated centre of *Polyorchis* to contract rhythmically, just as Loeb showed was true for the saline purgatives in the case of *Gonionemus*. It is, however, doubtful whether this throws any light on the way in which the vegetable purgatives act. It may be said in a general way that removing the margin of *Polyorchis* removes the nervous system and leaves the isolated centre relatively free from nervous elements. There is however no good proof that the centre is absolutely free from nerve cells or fibers. We cannot, therefore, absolutely state that the vegetable purgatives act on the muscular tissue alone in producing these contractions, although such a thing may seem probable.

ON THE SO-CALLED COFERMENT OF LIPASE.

(First Paper.)

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(Received for publication, November 13, 1906.)

In 1904 Magnus[1] investigated the action of liver lipase on amyl salicylate. He employed a clear extract of beef liver prepared by a modification of Jacoby's uranyl acetate method and made the interesting observation that its action on amyl salicylate depends upon the presence of two substances in the extract. One of these substances Magnus found to be of the nature of an enzyme, that is, it is non-dialyzable and is destroyed by boiling, while the other is dialyzable and is not destroyed by boiling. Magnus reached these conclusions as follows: The clear liver extract was dialyzed for several days against running water, when it was found that it had lost its activity. The activity returned, however, on adding to the dialyzed extract a quantity of boiled liver extract which had not been submitted to dialysis. Magnus was unable to determine the nature of the substance contained in the boiled extract which brings about the activation of the dialyzed extract. He investigated its properties, however, and found that it is soluble in water and in absolute alcohol, insoluble in ether, and that it is destroyed by ashing. He tried to activate the dialyzed extract by adding sodium chloride, leucin, tyrosin, glycocoll, lecithin, or bilirubin, but all failed to activate it. Magnus classified the unknown substance as a coferment.

The properties of the coferment led the writer to suspect that the bile salts are the essential constituents in the boiled extract, and on trial it was found that a solution of the bile salts really causes a return of activity to the dialyzed extract.

[1] *Zeitschr. f. physiol. Chem.*, xlii, p. 149, 1904.

A preliminary report of these researches with the bile salts was made at the last meeting of the American Physiological Society,[1] and Magnus,[2] in a paper which has just appeared, confirms my results. The bile salts used were prepared in the usual way from ox bile. In order to free the preparation of lecithin it was finely pulverized and extracted with ether for several hours in a Soxhlet apparatus. A large amount of the bile salts was smelted with the usual oxidation mixture and the residue tested for phosphate. The preparation contained but a trace of phosphorus. Clear solutions of lipase were prepared from beef liver in exactly the manner described by Magnus. While the extracts which we obtained were not quite as active as his, the results indicate that the bile salts constitute the coferment with which we have here to deal. For the following experiments 400 c.c. of clear liver extract were prepared and divided into two portions, one being placed in a parchment dialyzing tube and immersed in running water while the other portion was placed in a flask and immersed in the same water. Thus the two portions were kept under identical conditions except that one was dialyzing while the other was not. The dialysis was continued for eleven days, when the following experiments were performed:

(1) 20 c.c. extract (not dialyzed)
5 c.c. water

(2) 20 c.c. dialyzed extract
5 c.c. water

(3) 20 c.c. dialyzed extract
5 c.c. boiled extract (not dialyzed)

(4) 20 c.c. dialyzed extract
5 c.c. 0.2 per cent. solution of the bile salts

In addition to the above, each flask received 0.2 c.c. of toluene and 0.5 c.c. of amyl salicylate. The flasks were then corked and shaken at intervals. After remaining in the thermostat at 40° C. for six days salicylic acid was isolated from the

[1] *Amer. Journ. of Physiol.*, xv, p. xxvii, 1906.
[2] *Zeitschr. f. physiol. Chem.*, xlviii, p. 376, 1906.

mixtures by the usual method and determined colorimetrically with ferric chloride. The results were as follows:

(1)	salicylic acid found,	2.1 mg.
(2)	" " "	0.0 "
(3)	" " "	8.3 "
(4)	" " "	8.4 "

Other experiments were performed with similar results.[1] The results leave little room for doubt that the bile salts here constitute the coferment, and this conclusion is confirmed by the fact that the bile salts have practically all the properties which Magnus ascribes to the coferment.

Since the bile salts have been found to be the coferment in the reaction under consideration, the question naturally presents itself whether the term coferment is a useful one. Bertrand[2] introduced the term in connection with his work on laccase, the action of which was much increased by the presence of manganese salts. Hardin and Young[3] described a coferment for zymase. They pointed out later the importance of the alkaline phosphates present in their coferment solution. Buchner and Antoni[4] concluded that the action of Hardin and Young's coferment solution depends entirely upon the phosphoric acid which it contains and on the dilution of the sugar and alcohol by the boiled solution.

If we understand by the term coferment any substance, determined or undetermined, which renders possible the action of a ferment on any substance the term is a useful one. Under this definition acids are coferments for pepsin and the bile salts for the action of liver extract on amyl salicylate. It seems to the writer that the term coferment should not be applied to a substance which merely accelerates an enzymic process, as the term accelerator can be here used, but that it should be reserved for those substances which are absolutely necessary in order that the enzyme manifest itself at all. According to this conception the

[1] In this series it will be noted that less salicylic acid was formed in (1) than in (3). This was an isolated observation in this respect.

[2] *Compt. rend. de l'Acad. des sci.*, cxxiv, p. 1032, 1897.

[3] *Journ. of Physiol.*, xxxii, *Proc. of the Physiol. Soc.*, p. i, 1905; *Proc. of the Chem. Soc.*, xxi, p. 189, 1905.

[4] *Zeitschr. f. physiol. Chem.*, xlvi, p. 136, 1905.

coferments do not comprise a group of similar substances having many properties in common, as in the case of the enzymes, but on the contrary they constitute a very heterogeneous group including substances utterly dissimilar in their chemical nature and properties. Thus "enterokinase" and "thrombokinase," which are destroyed by heat, might fall into this group of coferments.

We have thus been able to confirm the results of Magnus on the action of liver extract on amyl salicylate, but when ethyl butyrate is used entirely different results are reached. It has repeatedly been observed in the course of this work that the action of the clear liver extract on ethyl butyrate is actually increased after prolonged (eleven days) dialysis. This is not invariably observed, however, and in most cases the extract does not alter materially in strength. Bile salts do not accelerate the action of this dialyzed extract on ethyl butyrate. We have therefore a striking difference between the hydrolysis of ethyl butyrate and amyl salicylate by liver extract. The fact brought out by Magnus is by no means of general application and the bile salts, far from increasing the action of the clear liver extract on other esters, are generally found to materially inhibit the process. The effect of bile salts on the hydrolysis of other esters will be considered in detail in a subsequent communication and also the question of the identity or non-identity of the enzymes which hydrolyze these esters. It is clear from the experiments quoted that the bile salts act as coferments only in the hydrolysis of amyl salicylate and not in the hydrolysis of esters—viz., in the action of lipase—in general. We have not determined the part which the bile salts play in the hydrolysis. It is to be observed, however, that amyl salicylate is a very insoluble ester and the bile salts may possibly act by increasing its solubility.

SUMMARY.

(1) We have been able to confirm the observations of Magnus that there exists a coferment for the action of liver extract on amyl salicylate.

(2) We have found the coferment to be the bile salts.

(3) When ethyl butyrate is used instead of amyl salicylate no coferment is to be noted by this method and the bile salts

do not accelerate this reaction. Therefore it is not justifiable to apply the term coferment to the bile salts with reference to the action of lipase in general. It must be stated that they have only been proven to act as such in the hydrolysis of amyl salicylate by liver extract.

Without postulating the identity or non-identity of the various ester-splitting enzymes, we may say that the bile salts constitute the coferment of hepatic amyl salicylase. This is merely a convenient form of expressing the experimental results.

Since this paper was written another communication from Hardin and Young has appeared[1] in which they prove the existence of a coferment for zymase.

[1] *Proc. of the Royal Soc.*, Series B, lxxviii, p. 369, 1906.

THE INHIBITING EFFECT OF SODIUM FLUORIDE ON THE ACTION OF LIPASE.

(Second Paper.)

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(Received for publication, November 13, 1906.)

Kastle and Loevenhart[1] first noted the remarkable inhibiting action of hydrofluoric acid and sodium fluoride on the ester-splitting property of liver and pancreas extracts. Hanriot[2] had previously stated that sodium fluoride does not inhibit the action of lipase on monobutyrin. Arthus[3] has since confirmed the statement of Kastle and Loevenhart that sodium fluoride inhibits the action of lipase. He found that the action of blood serum on monobutyrin is considerably retarded by this salt. In their experiments Kastle and Loevenhart studied the effect of sodium fluoride on the hydrolysis of ethyl acetate and ethyl butyrate by turbid liver and pancreas extracts. The experiments with ethyl butyrate and liver extract were of fifteen minutes' duration; the fluoride and hydrofluoric acid acted in a dilution of 1 to 5000. The per cent. hydrolysis was decreased from 8.54 to 1.13 by the sodium fluoride and from 8.54 to 0.63 by the hydrofluoric acid. In connection with other work to be presented later, we were led to study more closely the inhibiting action of sodium fluoride on the hydrolysis of fats and lower esters with the result that our observations seemed sufficiently interesting to warrant separate publication.

Experimental Methods.—Unless otherwise stated the enzymic preparations used in this work were from the liver and pancreas of the pig. In a few instances corresponding preparations from the dog were employed. We have used: (1) turbid extracts of

[1] *Amer. Chem. Journ.*, xxiv, p. 491, 1900.
[2] *Arch. de physiol.*, x, p. 797, 1898.
[3] *Journ. de physiol.*, iv, p. 56. 1902.

these organs prepared by grinding 10 grams of the fresh tissue with coarse white sand and extracting with water. This was then strained through cloth and the total volume made up to 100 c.c. Toluene was always added as the preservative. This preparation we shall refer to as 10 per cent. turbid extract. (2) Clear liver extracts prepared as follows: The turbid liver extract just described was placed in the thermostat at 40° C. for about eighteen hours, when coagulated masses appeared. It was then repeatedly filtered through an ordinary pleated filter, when a perfectly clear highly active filtrate is invariably obtained. The color of this filtrate varies from a straw color to bright red. We shall refer to this undiluted preparation as clear 10 per cent. liver extract, and in experiments in which it was diluted the degree of dilution will be indicated by the per cent. The corresponding preparation from the pancreas has only very slight activity, the enzyme apparently remaining in the coagulum. We have obtained clear extracts of the pancreas by the method proposed by Kastle, Johnston, and Elvove[1] for obtaining clear extract of the liver. One gram of fresh pig pancreas was ground up with sand and extracted with 75 c.c. of water. Then 2 c.c. of $\frac{N}{20}$ sodium hydroxide were added and the mixture warmed on the water bath to 38° C. when 3.5 c.c. of $\frac{N}{20}$ butyric acid were added. This causes a coagulum to separate and on filtering a very faintly opalescent filtrate is obtained. While the filtrate has some activity it is very decidedly less active than the turbid extract. The experiments were conducted with ethyl acetate, ethyl butyrate, diacetin, triacetin, and olive oil. The first four were employed in such quantities that for complete saponification they would require approximately 40 c.c. of $\frac{N}{20}$ sodium hydroxide. That is to say they were employed in acid-equivalent but not in molecular-equivalent quantities. This was done because Kastle and Loevenhart[2] found the hydrolysis to be largely independent of an excess of the ester, and furthermore it has been repeatedly shown that the acid produced inhibits further hydrolysis. For these reasons the esters were employed in quantities which would yield equivalent amounts of acid on complete hydrolysis. The

[1] *Amer. Chem. Journ.*, xxxi. p. 521. 1904.
[2] *Loc. cit.*

quantities actually used were as follows: ethyl acetate—0.1747 gram, ethyl butyrate—0.23 gram, diacetin—0.1714 gram, triacetin—0.1497 gram. Three-tenths of a gram of neutral olive oil was used in each experiment unless otherwise stated. This amount would require 21.36 c.c. of $\frac{N}{20}$ potassium hydroxide to saponify it. The titrations in the experiments with the lower esters were made with $\frac{N}{20}$ aqueous sodium hydroxide. In some experiments litmus was used as the indicator, in others phenolphthalein, no difference between the two being noted in the experiments with clear extracts. When turbid extracts were employed, litmus was always used. In the experiments with olive oil 20 c.c. of neutralized 85 per cent. alcohol, made up from absolute alcohol, were added at the conclusion of the incubation and the titration carried out with $\frac{N}{20}$ alcoholic potassium hydroxide, phenolphthalein being used as the indicator. All the experiments were carried out in the thermostat at 38°–40° C. Toluene was used as the antiseptic. Blanks of two kinds were carried through with each series: (1) Experiments with the active enzyme without the addition of ester; (2) experiments with the boiled extracts to which the ester was added. The initial acidity has always been deducted in the numbers given. The amount of acid formed in these blanks was insignificant except in one case which will be pointed out later.

The numbers given in the tables which follow state the amount of $\frac{N}{20}$ alkali in cubic centimeters required to neutralize the acid produced by the action of the enzyme on the ester. All necessary deductions have been made. The duration of the experiments varied and will be given in connection with each series. As will be seen in the tables, duplicate experiments were carried through in many instances. The averages of identical experiments are given and these were used in the calculation of the inhibition or acceleration. In the tables the concentration of the sodium fluoride in the total mixture is given.

EFFECT OF SODIUM FLUORIDE ON THE HYDROLYSIS OF OLIVE OIL AND ETHYL BUTYRATE BY 10 PER CENT. TURBID PANCREAS EXTRACT.

Series I.—Duration of experiments, 20 hours. Temperature, 38° C. One c.c. of a 10 per cent. turbid pancreas extract and 4 c.c. of water,

or 3 c.c. of water and 1 c.c. of sodium fluoride solution were used in each experiment.

The total volume in each case therefore was 5 c.c.

No. of Experiment.	Concentration of Na F.	Olive Oil. Grams.	Increase in Acidity.			Per cent. of Inhibition.
			(1)	(2)	Average.	
1	0.0	0.0	6.7	—	6.7	—
2	0.0	0.3	9.55	9.30	9.42	—
3	1:50,000	"	9.25	8.75	9.00	4.5
4	1:5,000	"	8.50	7.90	8.20	13.0
5	1:500	"	6.65	6.25	6.45	31.5
6	1:250	"	6.30	5.95	6.12	35.0

In Experiment 1 of the above series the pancreas extract showed a large production of acid on standing even when no olive oil was added. This has always been observed. It is undoubtedly to be attributed to the hydrolysis of the fat of the pancreas itself, which is invariably present in the turbid extract. This large production of acid in the turbid pancreas extract is not observed in aqueous titrations and is therefore due to the higher fatty acids. As shown in Experiments 2 and 3, the acid production was considerably increased by the addition of olive oil. Furthermore, the acid production is decreased by the presence of sodium fluoride, 1:250 (Exp. 6), to a point below that observed in Experiment 1 in which neither olive oil nor fluoride was added. It will be observed that the duplicate experiments do not show a perfect agreement. This is probably due to the fact that in measuring these turbid extracts, in spite of thorough shaking before each filling of the pipette unequal amounts of the suspended material were obtained. The agreement is sufficiently close, however, for our purpose. Although the differences between some of the duplicates are nearly as great as the difference seen with different concentrations of sodium fluoride, yet the averages show distinctly that with increasing dilution the inhibiting action of the fluoride gradually decreases. When the sodium fluoride acted in a concentration of 1:50,000 (Exp. 3) practically no inhibition (4.5 per cent.) was noted. It was very important for our purpose to make an accurate comparison of the inhibitory effect of sodium fluoride on the hydrolysis of olive oil and ethyl butyrate under conditions as nearly identical as

possible. It was necessary in making this comparison to use an extract which has considerable action on both of these substances. As will be pointed out later liver extract has very little action on olive oil, whereas pancreas extract is quite active on both. Therefore, two series (II and III) of experiments were carried through simultaneously which were identical in all respects except that olive oil was used in one series, while ethyl butyrate was employed in the other. It was necessary to use the same extract for both series because the effect of the fluoride varies to a certain extent with different extracts.

Series II.—Duration of experiments, 20 hours. Temperature, 38° C. Two c.c. of a turbid 10 per cent. pancreas extract and 3 c.c. of water, or 2 c.c. of water and 1 c.c. of sodium fluoride solution, were used in each experiment. The total volume in each case, therefore, was 5 c.c.

No. of Experiment.	Concentration of Na F.	Olive Oil. Grams.	Increase in Acidity.			Per cent. of Inhibition.	Per cent. of Acceleration.
			(1)	(2)	Average.		
1	0.0	0.0	3.1	3.15	3.12	—	—
2	0.0	0.3	6.15	6.10	6.12	—	—
3	1:5,000,000	"	6.90	6.95	6.92	—	13
4	1:500,000	"	6.30	6.90	6.60	—	8
5	1:50,000	"	6.05	6.80	6.42	—	5
6	1:5,000	"	5.20	6.65	5.92	3	—
7	1:500	"	3.10	3.05	3.07	50	—

We wish to emphasize that we do not attach much significance to small results as the method is hardly trustworthy for small differences. There is no doubt, however, that the fluoride, 1:5,000,000 (Exp. 3), accelerated the process. We have frequently seen such accelerations when very small amounts of the fluoride are used. Similar results have been obtained with the esters as will be pointed out. It has been observed by various investigators that in many instances a substance which in ordinary concentrations exerts a powerful retarding influence on a process, may in great dilution accelerate it. This has been observed with remarkable frequency and the case under consideration is no exception to the rule. This fact deserves recognition.

Series III.—Conditions identical in all respects to Series II, except that ethyl butyrate was used instead of olive oil.

No. of Experiment.	Concentration of Na F.	Increase in Acidity.			Per cent. of Inhibition.
		(1)	(2)	Average.	
1	0.0	9.58	8.98	9.28	—
2	1:5,000,000	9.13	9.03	9.08	2
3	1:500,000	9.03	8.53	8.78	5
4	1:50,000	7.53	7.78	7.65	18
5	1:5,000	2.93	2.73	2.83	70
6	1:500	1.38	1.38	1.38	85

The comparison of Series II with Series III, in the following summary, shows that the action of turbid pancreas extract on ethyl butyrate is inhibited to a much greater extent by the fluoride than is the action on olive oil.

	Inhibition.				
Na F	1:5,000,000	1:500,000	1:50,000	1:5,000	1:500
Olive oil	0.0.	0.0	0.0	2 per c't	50 per c't
Ethyl butyrate	2 per cent.	5 per cent.	18 per cent.	70 per c't	85 per c't

Thus the concentration, 1:5,000,000, was approximately the vanishing point of the inhibition with the butyrate, while 1:5,000 represented the same point with the olive oil. Viewed in this way therefore, the inhibiting effect of the fluoride on the hydrolysis of the butyrate is about one thousand times as great as on the hydrolysis of olive oil. In some experiments with a specially prepared pancreas powder we have been able to inhibit the action on ethyl butyrate to the extent of 85 per cent. by means of sodium fluoride 1:5,000, while the action on olive oil showed no inhibition whatever under the same conditions.

EFFECT OF SODIUM FLUORIDE ON THE HYDROLYSIS OF ESTERS BY CLEAR LIVER EXTRACT.

The action of clear liver extract on the lower esters proved to be even more sensitive to the fluoride than the action of the turbid extracts of the pancreas.

Series IV.—Duration of experiments, 17 hours. Temperature, 40° C. One c.c. of clear 10 per cent. extract of pig liver, 0.23 gm. of ethyl butyrate, and 4 c.c. of water, or 3 c.c. of water and 1 c.c. of sodium fluoride solution, were used in each experiment.

No. of Experiment.	Concentration of Na F.	Increase in Acidity. (1)	(2)	Average	Per cent. of Inhibition.
1	0.0	8.43	8.47	8.45	—
2	1:5,000,000	7.95	8.25	8.15	3.5
3	1:500,000	3.85	3.70	3.78	55
4	1:50,000	1.08	1.07	1.08	87

Thus the fluoride when acting in a concentration of 1:500,000 depresses the actions over 50 per cent. but at a dilution of 1:5,000,000 very little inhibition was noted in this series The effect of the fluoride 1:5,000,000 varied in different series. In some cases it caused an inhibition of as high as 11 per cent., while in one instance it caused an acceleration of 6 per cent. The action of the fluoride varies considerably with different esters of the lower fatty acids. In this connection we have studied the behavior towards ethyl acetate, diacetin, and triacetin.

Series V.—Ethyl acetate. Experiments were performed in manner described in previous series. Duration, 18 hours at 40° C.

No. of Experiment.	Concentration of Na F.	Increase in Acidity. (1)	(2)	Average.	Per cent. of Inhibition.
1	0.0	4.43	4.19	4.31	—
2	1:50,000,000	3.94	3.95	3.95	8
3	1:5,000,000	2.23	2.08	2.16	50
4	1:500,000	0.65	0.60	0.63	85
5	1:50,000	0.15	0.15	0.15	96.5
6	1:5,000	0.10	0.13	0.12	97.2

Series VI.—Diacetin. Otherwise, same as above.

No. of Experiment.	Concentration of Na F.	Increase in Acidity. (1)	(2)	Average.	Per cent. of Inhibition.
1	0.0	2.4	2.2	2.3	—
2	1:500,000,000	2.41	1.97	2.19	5
3	1:50,000,000	2.19	1.80	2.00	13
4	1:5,000,000	1.22	1.40	1.31	43

Series VII.—Triacetin. Conditions same.

No. of Experiment.	Concentration of Na F.	Increase in Acidity.			Per cent. of Inhibition.
		(1)	(2)	Average.	
1	0.0	2.4	2.6	2.5	—
2	1:50,000,000	2.1	2.68	2.39	4
3	1:5,000,000	1.67	1.50	1.58	37

The last three series of experiments show that these three esters of acetic acid show about the same degree of inhibition. It seems to make little difference whether the combined alcohol is ethyl alcohol or glycerin. On comparing them with ethyl butyrate (Series IV) it will be seen that the hydrolysis of the latter is inhibited to a much less extent than the esters of acetic acid. The inhibition with acetic esters is about ten times as great as with the butyrate ester calculated from the values found with sodium fluoride, 1:5,000,000.

In order to determine whether the inhibition is influenced by the proportion of fluoride to enzyme, a series was carried out in which the amount of enzyme varied, while the concentration of the fluoride in all the tubes was the same, viz., 1:100,000.

Series VIII.—Duration of experiments, 24 hours. Temperature, 40° C. Ten per cent. clear extract of pig liver, ethyl butyrate, Na F in every experiment, 1:100,000. The total volume was 5 c.c. in each experiment.

No. of Experiment.	Liver Extract.	Na F.	C.C. of $\frac{N}{20}$ NaOH Required.			Per cent. of Retardation.
			(1)	(2)	Average.	
1	4	—	11.97	12.32	12.14	—
2	4	+	5.87	5.77	5.82	52
3	2	—	10.41	10.56	10.48	—
4	2	+	3.26	3.06	3.16	70
5	1	—	8.48	8.43	8.45	—
6	1	+	1.86	1.86	1.86	78
7	$\frac{1}{4}$	—	5.97	5.87	5.92	—
8	$\frac{1}{4}$	+	0.52	0.52	0.52	91.2
9	$\frac{1}{16}$	—	3.89	3.69	3.79	—
10	$\frac{1}{16}$	+	0.14	0.19	0.16	95.8

From this series of experiments we see that for a given strength of sodium fluoride the smaller the amount of enzyme acting the greater is the percentage inhibition. This proves that there

exists a quantitative relationship between the enzyme and the fluoride. We will return to the significance of this result later.

By using very dilute liver extract it is possible to show that the fluoride, 1: 100,000,000, exercises a distinct inhibiting action on the process, as shown in the following.

Series IX.—Duration of experiments, 20 hours. Temperature, 39° C. One c.c. of 10 per cent. clear liver extract was diluted to 16 c.c. with water and 1 c.c. of this was used in each experiment together with ethyl butyrate, and 4 c.c. of water, or 3 c.c. of water and 1 c.c. of sodium fluoride.

No. of Experiment.	Concentration of Na F.	C.C. of $\frac{N}{20}$ NaOH Required.				Per cent. of Retardation.
		(1)	(2)	(3)	Average.	
1	0.0	5.20	5.13	5.05	5.13	—
2	1:1,000,000,000	5.08	5.10	5.11	5.10	0.6
3	1:100,000,000	4.95	4.68	5.02	4.88	4.9

Another series of experiments was performed with even more dilute liver extract.

Series X.—Duration of experiments, 17 hours. Temperature, 41° C. One c.c. of 10 per cent. clear liver extract was diluted to 100 c.c. with water and 1 c.c. of this was used in each experiment together with ethyl butyrate and 4 c.c. of water, or 3 c.c. of water and 1 c.c. of sodium fluoride.

No. of Experiment.	Concentration of Na F.	C.C. of $\frac{N}{20}$ NaOH Required.			Per cent. of Acceleration.
		(1)	(2)	Average.	
1	0.0	1.10	0.95	1.02	—
2	1:1,000,000,000	1.31	1.34	1.32	29
3	1:100,000,000	1.13	0.97	1.05	3

Thus with this very dilute extract the fluoride, 1: 1,000,000,000, caused a remarkable acceleration in the process. We have already pointed out that the fluoride when acting in very dilute solution also accelerates the action on olive oil, but the amount required to show the acceleration with olive oil would be sufficient to very greatly inhibit the action on the lower esters.

It was frequently noted after titrating a series that those tubes which contained the fluoride developed acid much faster than the

corresponding tubes in which no fluoride was present. Thus Tubes 3 and 4 of Series VIII were allowed after titration to stand one hour at room temperature when they were again titrated.

No. 3 (no fluoride) required 0.55 c.c. of $\frac{N}{20}$ NaOH
No. 4 (fluoride, 1:100,000) required 3.10 c.c. of $\frac{N}{20}$ NaOH.

This fact proves that the fluoride does not destroy the enzyme but merely inhibits its action.[1] It has been shown repeatedly that the enzyme is destroyed by acids, and since the tubes containing no fluoride developed such a large amount of acid during the incubation, the enzyme in these tubes was largely destroyed. The fluoride by inhibiting the action of the enzyme decreased the amount of acid to which the enzyme was exposed during the incubation and thus indirectly conserved it. This is probably the principal reason why the fluoride tubes show a greater acid production than the water tubes after the titration. We found, however, that there is at least one other important factor which must be taken into consideration here. It is this: The inhibiting action of the fluoride increases as acid develops. This was shown by the following experiments:

(1) 1 c.c. of clear 10 per cent. extract of pig's liver.
4 c.c. of water.
Ethyl butyrate.
Litmus.

(2) Exactly the same as No. 1.

(3) 1 c.c. of the same liver extract.
3 c.c. of water.
1 c.c. of sodium fluoride (1:10,000).
0.26 c.c. of ethyl butyrate.
Litmus.

(4) Exactly the same as No. 3.

Tubes 1 and 3 were kept constantly very near the neutral point by adding $\frac{N}{20}$ NaOH as rapidly as they became acid. Tubes 2 and 4 were

[1] Note added during proofreading.—We have found that the fluoride may be completely removed from the extract by dialysis leaving the enzyme uninjured. The activity after dialysis was as great as that of the extract which had not been treated with the fluoride.

allowed to stand at room temperature. All of the experiments lasted 36 minutes. The results were as follows:

					Remarks.	Per cent. of Inhibition.
(1)	required	5.02	c.c.	$\frac{N}{20}$ NaOH.	Kept at neutral point.	—
(3)	"	2.62	"	"	" " " "	47.6
(2)	"	1.70	"	"	Acid allowed to accumulate.	
(4)	"	0.35	"	"	" " " "	79.4

Thus the tubes which were kept as nearly neutral as possible showed much less percentage inhibition than those in which the acid was allowed to accumulate. Hence the inhibiting action of the fluoride becomes greater as acid develops. The probable explanation of this is that hydrofluoric acid is a more powerful inhibitor than sodium fluoride and as the solution becomes more acid more hydrofluoric acid is produced. The fact that an inhibition of 47.6 per cent. was noted even when the mixture was kept neutral proves, however, that sodium fluoride is a powerful inhibitor in itself entirely independent of the production of hydrofluoric acid. Hydrofluoric acid is quite weak and even the organic acids can decompose sodium fluoride with the production of hydrofluoric acid. The difference observed in the inhibiting effect of the fluoride on different esters may be in part attributed to variations in the amount of hydrofluoric acid produced by the acids resulting from the hydrolysis. We are sure, however, that this does not explain the very great difference noted between the esters of the lower and higher fatty acids because even when the mixtures were kept neutral in the above experiments, the fluoride 1: 50,000 caused an inhibition of 47.6 per cent. whereas sodium fluoride 1:50,000 only very slightly inhibits the action on olive oil.

Three facts prove conclusively that the inhibition caused by the fluoride is in no way connected with the precipitation of calcium, viz: (1) The amount of fluoride required is so small that at such dilution calcium fluoride is soluble: (2) Ammonium oxalate does not inhibit the reaction to any extent: (3) The addition of calcium chloride does not decrease the inhibiting action of the fluoride.

In order to be sure that the inhibiting action possessed by the fluoride is not shared by other salts, a series was carried

through with the other halides of sodium and potassium and with several other substances.

Series XI.—Duration of experiments, 21 hours. Temperature, 38° C. One c.c. of a 10 per cent. clear extract of dog liver, ethyl butyrate, and 4 c.c. of water, or 3 c.c. of water and 1 c.c. of the solution (1:250), were used in each experiment. The substance therefore acted in a concentration of 1:1250.

No. of Experiment.	Substance.	$\frac{N}{20}$ NaOH Required. C.C.	Per cent. of Retardation.	Per cent. of Acceleration.
1	Water	5.12	—	—
2	$NaCl$	4.35	15	—
3	$NaBr$	5.25	—	3
4	NaI	4.30	16	—
5	KCl	4.40	14	—
6	KBr	4.65	9	—
7	KI	4.20	18	—
8	$NaNO_3$	4.05	21	—
9	KNO_3	4.05	21	—
10	$CaCl_2$	4.15	19	—
11	$BaCl_2$	4.70	8	—
12	$MnCl_2$	4.70	8	—
13	$CdCl_2$	3.50	32	—
14	Na_2HPO_4	6.50	—	27
15	$Na_2Fe(CN)_5NO$	2.50	51	—
16	K_2CrO_4	5.75	—	12
17	NH_4CNS	2.75	46	—
18	$CS(NH_2)_2$	5.20	—	2

Thus we have been unable to find any substance which exercises an effect on the reaction which is comparable to that of the fluoride. Several interesting facts are brought out in the table, however. A comparison of the halides of sodium and potassium shows that in both cases the bromide inhibits the reaction less than the chloride or iodide. In the case of halides and the nitrate practically no difference is seen between the sodium and potassium salts. The chlorides of calcium, barium, manganese, and cadmium all inhibit the process. The results obtained with ammonium sulphocyanate and thiourea are interesting. The former exercises a marked inhibiting action, while the latter is practically without effect. Kastle and Loevenhart[1]

[1] *Amer. Chem. Journ.*, xxix, p. 397, 1903

found exactly the same thing in their work on liver catalase. Here also the sulphocyanate exercised a marked inhibiting effect, while its isomer, thiourea, somewhat accelerated the decomposition.

Thus the action of sodium fluoride[1] is perfectly characteristic as far as we have been able to determine and the question presents itself, What is the mode of action of the fluoride? Does it act on the enzyme or on the ester? It is impossible, in the present state of knowledge of enzyme action, to give all the possible ways in which an enzymic process may be affected by a substance. We may say, however, in a general way that there are at least five general modes of action by which a foreign substance (*i.e.* a substance which is not essential to the process or a product of the reaction) may accelerate or retard an enzymic process. (1) It may react with the enzyme. The inhibiting and destructive action of acids, in general, on animal lipase is an instance of this kind. (2) It may react with the substance upon which the enzyme acts, the zymolyte.[2] An instance of this kind is the retarding action of formaldehyde on the coagulation of milk by rennin. Bliss and Novy[3] found that rennin is not affected by formaldehyde but that this substance acts on the caseinogen changing it to a body which is not readily coagulated by rennin. (3) It may react with one of the products resulting from the process and remove it from the field of action. An excellent example of this is the accelerating effect of calcium salts on the action of rennin. The calcium salts precipitate the paracasein as rapidly as it is formed. (4) The solubility of the enzyme or the zymolyte may be altered. (5) The possibility exists that the substance may react with an intermediate compound formed by

[1] Hydrofluoric acid and in all probability all soluble fluorides would act similarly.

[2] It is necessary to have some general term to designate the substance upon which any enzyme acts. The Germans have introduced the term "substrat" and this has recently been used by English and American writers in the forms substrate or substratum. There is certainly no logical justification for this term. We propose the term zymolyte because it is in keeping with general chemical nomenclature and signifies definitely what is meant. It corresponds to "electrolyte," etc.

[3] *Journ. of Exper. Med.*, iv, p. 47, 1899.

the action of the enzyme on the zymolyte in consequence of which its stability may be increased or decreased.

Kastle and Loevenhart[1] found that hydrocyanic acid accelerates the decomposition of hydrogen peroxide by copper sulphate, and Loevenhart[2] showed that hydrocyanic acid accelerates the oxidation of formic acid by hydrogen peroxide in the presence of copper sulphate. The probable explanation of both of these facts is that the hydrocyanic acid facilitates in some way the formation and decomposition of intermediate products formed in the reaction.

In order to be sure that the fluoride does not react in some way with the ester in consequence of which the stability of the latter might be increased, a series of experiments was performed to determine the effect of fluoride on the hydrolysis of the esters by acids and alkalies. It was found that sodium fluoride does not inhibit the alkaline hydrolysis of ethyl butyrate, diacetin, and triacetin to any noteworthy extent if at all, and in the hydrolysis of these esters by hydrochloric acid it acts merely as the sodium salt of any weak acid should. That is to say, it retards the hydrolysis only by using up a part of the hydrochloric acid and replacing it with the much weaker hydrofluoric acid. Hence we are justified in stating that the fluoride probably does not inhibit the action of the enzyme by reacting with the ester.

It is obvious that the fluoride does not inhibit the process by reacting with either of the products or by altering the solubility of the enzyme or ester. Therefore but two of the five possibilities remain. Either it must react with the enzyme or with some intermediate product. We have pointed out that it certainly does not destroy the enzyme. Furthermore, if it causes the inhibition by reacting with the enzyme, we would expect it to retard the hydrolysis of all the esters to a proportionate degree. But we have found large variations when different esters are used. These facts certainly speak against the view that the fluoride reacts with the enzyme.

We have found, however, that the effect of the fluoride increases

[1] *Amer. Chem. Journ.*, xxix, p. 397, 1903.

[2] *Ber. d. deutsch. chem. Gesellsch.*, xxxix, p. 130, 1906.

as the amount of enzyme acting decreases. While we cannot be sure, we favor the view that it reacts with an intermediate product formed by the action of the enzyme on the ester in consequence of which the intermediate product is rendered more stable. This view seems to us to be more in harmony with all the facts which we have brought out.

In general chemical behavior the fluorides differ widely from the other halides. Corresponding to this chemical dissimilarity the pharmacological action of sodium fluoride differs entirely from that of the other halides. It is highly poisonous to nearly all forms of life, the fatal subcutaneous or intravenous dose being put at 0.15 gram per kilo for dogs.[1] Its pharmacological action is doubtless very complicated. Some of its effects are probably due to the removal of calcium, but this alone apparently cannot fully explain its action. The remarkable inhibiting action of sodium fluoride on the enzymic hydrolysis of esters is the more striking when it is remembered that it is ordinarily considered a protoplasmic poison like chloroform, toluene, etc., which does not interfere with enzymic processes.[2] Pavy[3] sounded a warning against this erroneous view and showed that sodium chloride in 1 per cent. solution inhibits the action of diastase on starch about 20 per cent. Its inhibiting action on the hydrolysis of esters, however, is incomparably greater than on any other enzymic process as far as we are aware, and the possibility naturally suggest itself that its toxic action may be due in part to the inhibition of this class of processes in the organism. It suggests the possibility that some cleavage of this type is of vital importance in the economy.

SUMMARY.

1. Sodium fluoride inhibits in a remarkable way the hydrolysis of esters by liver and pancreas extracts.

2. The hydrolysis of ethyl butyrate by pancreas extract is from one hundred to one thousand times as sensitive to the

[1] Tappeiner, *Arch. f. exper. Path. u. Pharm.*, xxv, p. 203, 1889; xxvii, p. 108, 1890.

[2] Arthus and Huber, *Arch. de physiol.*, 5e Ser. iv, p. 651, 1892.

[3] ...rn. *of Physiol.*, xxii, p. 391, 1898.

inhibiting action of the fluoride as is the hydrolysis of olive oil, by the same preparation and under the same conditions.

3. The action of clear liver extract on the lower esters is much more sensitive to the fluoride than is the action of turbid pancreas extract. Sodium fluoride, 1:500,000, inhibited the action of 2 per cent. clear liver extract to the extent of 78 per cent., while that of 2 per cent. turbid pancreas extract suffered an inhibition of only 5 per cent.

4. The hydrolysis of ethyl acetate is inhibited to a greater extent by the fluoride than that of ethyl butyrate. The fluoride inhibited the hydrolysis of ethyl acetate, diacetin, and triacetin to about the same extent. Hence the acid from which the ester is derived seems to be the principal factor in determining the extent of the fluoride inhibition. The inhibition seems to be largely independent of the alcohol from which the ester is derived.

5. Very small amounts of sodium fluoride (varying with different esters and extracts) accelerate the hydrolysis of the fats and lower esters by the extracts. Thus the fluoride, 1:1,000,000,000, caused an acceleration of 29 per cent. in the action of 0.02 per cent. liver extract on ethyl butyrate.

6. The inhibiting action of the fluoride increases as the tubes become more acid. This is probably due to the production of hydrofluoric acid.

7. Sodium fluoride does not influence materially, if at all, the hydrolysis of esters by alkalies and it only affects the hydrolysis of esters by acids as the sodium salt of any weak acid should. From this we conclude that the fluoride probably does not inhibit the enzymic hydrolysis of the esters by reacting with the latter.

8. The inhibiting action of the fluoride cannot be attributed to the precipitation of calcium.

9. The other halides of sodium and potassium as well as many other substances investigated do not possess an action comparable in extent to that of the fluoride. Hydrofluoric acid and in all probability all soluble fluorides affect these processes similarly to sodium fluoride.

10. The greater the amount of enzyme acting the less will be the inhibiting effect of a given concentration of sodium fluoride.

This proves that there exists a quantitative relation between the enzyme and the fluoride.

11. Sodium fluoride does not destroy the enzyme.

12. Of the two modes of action to be considered, (1) that the fluoride reacts with the enzyme, (2) that it reacts with an intermediate product formed in the action of the enzyme on the ester and resulting in an increase in the stability of the intermediate compounds, we favor the latter view, because the enzyme is not destroyed by the fluoride and furthermore because the inhibition varies to such an extent with different esters.

13. We suggest that the substance upon which an enzyme acts be called the "zymolyte," since this term is more in keeping with general chemical nomenclature and is preferable in every way to the term substrate which has recently been introduced.

ON THE EFFECT OF BILE UPON THE HYDROLYSIS OF ESTERS BY PANCREATIC JUICE.

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(Received for publication, November 13, 1906.)

The part played by the bile in the digestion and absorption of fats has long been a subject of discussion. It has been proved conclusively that the bile greatly promotes the absorption of fats and that in its absence fat absorption is much impaired. Although various views on fat absorption have been held by different investigators, all supposed that the bile aids the process by acting as a solvent for the fatty acids liberated by the action of lipase and by assisting in the emulsification of the neutral fat. Hewlett[1] has recently found that the pancreatic juice as secreted possesses comparatively little activity toward the esters of the lower fatty acids. He holds that the principle function of the bile is to render the lipase more active. He discusses briefly the mechanism by which the bile exercises this activating effect and concludes that some constituent of the bile acts as a zymo-exciter or accelerator for the lipase, but dismisses as improbable the view that the bile activates a lipase zymogen. Hewlett arrived at this conclusion by using triacetin instead of the higher fats. Triacetin is quite soluble in water, and, moreover, the products of its hydrolysis mix with water in all proportions. Hence, if bile is capable of accelerating the action of pancreatic juice on triacetin, its action in this case certainly cannot be explained by the solvent power of the bile on the zymolyte or on the products of the reaction. Hewlett found that bile greatly accelerates the action of pancreatic juice on triacetin. Thus, in the experiments which he quotes the bile increased the hydrolysis twenty-six times in the first hour, while at the end of twenty-four hours the hydrolysis was one and one-half times as great as in the absence of bile. Similarly the hydrolysis of ethyl butyrate was accelerated seven to sixteen times by

[1] *Johns Hopkins Hospital Bulletin*, xvi, p. 20, 1905.

the addition of bile. This very important contribution could only have been made by using such a water-soluble ester as triacetin. Hewlett sought to determine which constituent of the bile is responsible for this acceleration. He found that commercial lecithin is capable of accelerating the action of pancreatic juice to the same extent that the bile does. He found that a commercial preparation of the bile salts also accelerates the action of the pancreatic juice on the ester but as the bile salts are purified, their accelerating action decreases. He concluded that the accelerating action of the bile salts is probably due to a contamination with lecithin. The generally accepted view has been that the bile salts are the important constituents of the bile in assisting in the absorption of fats.[1] Strecker[2] first called attention to the fact that the bile salts increase the solubility of the higher fatty acids in water, and the experiments of Marcet[3] showed the solubility of fatty acids in bile and the emulsification of neutral fat by this mixture. Hence the bile salts have been looked upon as the active constituents of the bile in fat absorption. Martin and Williams[4] found that the bile salts accelerate the action of pancreatic disastase, and they attribute the accelerating action of the bile in this process to the bile salts.

We have found that results obtained in the action of lipase on one ester cannot be inferred to hold when another ester is used. Hence it becomes necessary to see whether the observations of Hewlett regarding the accelerating action of lecithin will hold when the higher fats are used. We have determined quantitatively the activity of pancreatic juice on several esters, including olive oil, in the presence and absence of bile salts, lecithin, and bile. In doing this we have had two objects in view: (1) to determine, if possible, which constituent of the bile under physiological conditions accelerates the action of pancreatic juice; (2) to determine whether the hydrolysis of the various esters is affected in the same way and to the same extent by these substances. Since the publication of a preliminary

1 Howell, *A Text-book of Physiology*, 1905, p. 715.
2 *Ann. d. Chem.*, lxv, p. 1, 1848.
3 *Proc. of the Roy. Soc.*, ix, p. 306, 1858.
4 *Ibid.*, xlv, p. 358, 1889.

communication by one of us[1] on the part played by the bile salts in the hydrolysis of amyl salicylate by liver extract, two articles have appeared on the subject. Von Fürth and Schütz[2] in a preliminary communication state that Plattner's bile salts possess the full activity of the bile in accelerating the hydrolysis of fat by pancreatic juice. They find, furthermore, that pure sodium glycocholate behaves similarly and that sodium cholate is also very active. They conclude therefore that the power of the bile to accelerate this process resides in the cholic acid complex of the bile salts. No data are given in this communication. Magnus[3] has found that the bile salts synthetically prepared, accelerate the action of pancreatic juice on olive oil.

Experimental Methods. The pancreatic juice was obtained from dogs by establishing a temporary fistula and injecting secretin. The bile was also collected in most cases, and it is interesting to note in this connection that the volumes of pancreatic juice and of bile collected in each experiment were usually practically equal. This would indicate that in the intestine the digesting fluid probably consists of about equal volumes of bile and pancreatic juice. The biliary canula was never forced as high up as the cystic duct. In some cases the bile was obtained from the gall bladder directly. The titrations were carried out in the manner described on p. 399 of the preceding article. The indicators were used as described in the preceding article, p. 399. Toluene was always used as the antiseptic. The usual blanks were carried through, and all necessary corrections for acidity or alkalinity have been made in the data which follow. The results are recorded in the number of cubic centimeters of $\frac{N}{20}$ alkali required to neutralize the acid produced by the enzymic hydrolysis of the ester. The esters investigated and the quantities used unless otherwise stated were as follows: ethyl acetate—0.1747 gram, ethyl propionate—0.2082 gram, ethyl butyrate—0.23 gram, diacetin—0.1714 gram, triacetin—0.1497 gram, and olive oil—0.3 gram. The reason for using the esters in these quantities is given on page 398 of the preceding

[1] *Amer. Journ. of Physiol.*, xv, p. xxvii, 1906.
[2] *Centralbl. f. Physiol.*, xx, p. 47, 1906.
[3] *Zeitschr. f. physiol. Chem.*, xlviii, p. 376, 1906.

article. The duration of the experiments and the temperature are given in connection with each series. We have studied the effect of bile salts, lecithin, mixtures of bile salts and lecithin, and bile on the hydrolysis of the above mentioned esters by the juice. The bile salts were prepared from ox bile and were purified by solution and reprecipitation and finally by extracting for several days with ether in a Sohxlet extractor. A large quantity was smelted with the usual oxidation mixture and showed only a trace of phosphorus. The lecithin used was prepared by the method of Roaf and Edie[1] and was employed in a 10 per cent. aqueous suspension. The bile was obtained from dogs, and in most cases from the animal that yielded the pancreatic juice of the experiment. In order to compare the bile with bile salts at various concentrations, the bile was assumed to contain 5 per cent. of bile salts and was diluted accordingly. Thus to compare bile with a 0.1 per cent. solution of the bile salts, the bile was diluted with 49 volumes of water. This will be designated "bile = bile salts 0.1 per cent," etc. In the tables that follow, the concentration of the bile salts and lecithin in the total mixture is given and not the concentration of the solution or suspension added. Thus in speaking of the action of 0.1 per cent. bile salts we mean that the bile salts were present in the total mixture in this concentration.

Experimental Results. We have been able to confirm the observations of Hewlett that lecithin accelerates the action of pancreatic juice on the lower esters, and we have found that it also accelerates the action of the juice on olive oil. We have found that the bile salts also greatly accelerate the action of pancreatic juice on all of the esters investigated. In order to bring out the accelerating action on the hydrolysis of the various esters, it is necessary to employ the bile salts in different concentrations. Thus for the lower esters the bile salts should be used in a concentration of from 0.13 to 0.05 per cent. In these concentrations they do not accelerate the hydrolysis of olive oil. When this latter ester is employed, the bile salts should be used in a concentration of from 2 to 4 per cent. All of these facts are brought out in the following tables.

[1] *Thompson-Yates and Johnston Lab. Rep.*, vi (1), p. 201, 1905.

Series 1. Duration of experiments, 16 hours. Temperature, 38° C. One c.c. of pancreatic juice was used in each experiment and this was diluted to 2.1 c.c. in every case.

No. of Experiment.	Ester.	Bile Salts. Per cent.	Lecithin Suspension. Per cent.	$\frac{N}{20}$ Alkali Required. c.c.	Per cent. of Acceleration.
1	Ethyl butyrate	0.00	0.00	3.2	—
2	" "	0.05	0.00	6.65	108
3	" "	0.00	0.5	6.75	111
4	Triacetin	0.00	0.00	9.05	—
5	"	0.05	0.00	9.30	3
6	"	0.00	0.5	10.25	13

Series 2. Duration of experiments, 18 hours. Temperature, 39° C. One c.c. of pancreatic juice was used in each experiment and this was diluted to 3 c.c. with water or lecithin suspension.

Ester.	Lecithin Suspension. Per cent.	$\frac{N}{20}$ Alkali Required. c.c.	Per cent. of Acceleration.
Olive oil	0.0	1.2	—
" "	0.7	7.2	500

Series 3. Duration of experiments, 16 hours. Temperature, 38° C. Similar in all respects to Series 2.

Ester	Lecithin Suspension. Per cent.	$\frac{N}{20}$ Alkali Required. c.c.	Per cent. of Acceleration.
Olive oil	0.0	1.25	—
" "	0.3	2.95	136

Series 4. Duration of experiments, 19 hours. Temperature 40° C. Two c.c. of pancreatic juice were used in each experiment and this was diluted to 3 c.c.

No. of Experiment.	Ester.	Bile Salts. Per cent.	Bile.	$\frac{N}{20}$ Alkali Required. c.c.	Per cent. of Acceleration.
1	Ethyl butyrate	0.0	—	1.50	—
2	" "	0.1	—	6.60	340
3	Diacetin	0.0	—	3.25	—
4	"	0.1	—	8.00	146
5	Triacetin	0.0	—	4.85	—
6	"	0.1	—	13.10	170
7	Olive oil	0.0	—	1.15	—
8	" "	0.17	—	1.25	9
9	" "	0.0	=bile salts 1.7 per cent.	13.00	1030

Series 5. Duration of experiments, 19 hours. Temperature, 35° C. One c.c. of pancreatic juice was used in each experiment and this was diluted in each case to 5 c.c.

No. of Experiment.	Ester.	Bile Salts. per cent.	$\frac{N}{20}$ Alkali Required. c.c.	Per cent. of Acceleration.	Per cent. of Inhibition.
1	Ethyl butyrate	0.0	1.90	—	—
2	" "	0.8	3.20	68	—
3	" "	4.0	2.35	24	—
4	Diacetin	0.0	3.25	—	—
5	"	0.8	2.95	—	9
6	Triacetin	0.0	5.80	—	—
7	"	0.8	3.60	—	38
8	"	4.0	3.85	—	34
9	Olive oil	0.0	3,60	—	—
10	" "	0.8	9.55	165	—
11	" "	4.0	10.85	201	—

Series 6. Duration of experiments, 22 hours. Temperature, 38° C. One c.c. of pancreatic juice was used in each experiment and this was diluted in each case to 5 c.c.

No. of Experiment.	Ester.	Bile Salts. Per cent	Bile.	$\frac{N}{20}$ Alkali Required. c.c.	Per cent. of Acceleration.	Per cent of Inhibition.
1	Ethyl butyrate	0.0		2.35	—	—
2	" "	0.1		3.90	66	—
3	" "	0.0	=bile salts 0.1 per cent.	4.45	89	—
4	" "	2.0		3.20	36	—
5	" "	4.0		3.35	43	—
6	Triacetin	0.0		9.80	—	—
7	"	0.1		8.00	—	18
8	"	4.0		4.25	—	57
9	Olive oil	0.0		4.25	—	—
10	" "	0.1		4.25	0.0	—
11	" "	0.0	=bile salts 0.1 per cent.	4.40	3.5	—
12	" "	2.0		10.10	138	—
13	" "	0.0	=bile salts 2 per cent.	18.00	324	—
14	" "	4.0		11.65	174	—

We have selected the foregoing experiments because they show the widest variations in the values which we have found. The pancreatic juice from different dogs does not give the same results. It will be seen in the tables that both lecithin and the

bile salts accelerate the action of the juice on olive oil and on the lower esters. The effect of the bile salts varies greatly with the concentration. Thus in a concentration of 0.1 per cent. the bile salts greatly accelerate the action on the lower esters and this acceleration decreases with increasing concentration. In a concentration of 0.8 to 4 per cent. they invariably inhibited the action of the juice on triacetin (Series 5). In Series 6, 0.1 per cent. bile salts inhibited the action on triacetin. This is occasionally seen. In Series 1, the bile salts, 0.05 per cent., cause practically no acceleration, while in Series 4 (0.1 per cent. bile salts) an acceleration of 170 per cent. in the hydrolysis of triacetin is noted. We have uniformly found that pancreatic juice without the addition of any accelerator is from 3 to 4 times as active on triacetin as on ethyl butyrate, and the accelerations which we have noted in experiments with triacetin are correspondingly less than with ethyl butyrate. In Series 4, where the activity on triacetin was less than usual the acceleration noted was greater. Experiments 8 of Series 4, 10 and 11 of Series 5, and 10 to 14 of Series 6, show that 0.1 per cent. bile salts do not accelerate the action of the juice on olive oil, but when present in a concentration of 0.8 to 4 per cent. a very great acceleration is noted. The same is true of bile. When the bile is so diluted that the bile salts are acting in a concentration of 0.1 per cent. practically no acceleration is noted (Exp. 11, Series 6).

On comparing the experiments in which bile salts and bile were used in similar concentrations, it will be seen that in many instances bile causes a greater acceleration than the bile salts. This is by no means invariably found to be the case as will be subsequently shown, but these experiments taken alone would naturally lead to the conclusion that both the bile salts and the lecithin or at least some other constituent of the bile may play a part in the accelerating action. Several series of experiments were performed in order to see whether mixtures of the bile salts and lecithin cause a much greater acceleration than these substances do when acting individually; in other words, to see whether they give a summation effect. We have carried on these experiments with a series of esters in order to see whether the hydrolysis of the different esters is affected to the same degree by these substances.

SERIES 7. Duration of experiments, 18 hours. Temperature, 39° C. One c.c. of pancreatic juice was used in each experiment. It was diluted to 5 c.c. in every case. The bile salts acted in a concentration of 0.1 per cent., the lecithin in a 0.4 per cent. suspension; the mixture which will be designated "bile salts + lecithin" contained 0.1 per cent. of the former and 0.4 per cent. of the latter, and the experiments designated "bile" contained 0.1 c.c. of bile from the same dog which, on the supposition that the bile contained 5 per cent. of bile salts would make the bile salts present in this experiment in a concentration of 0.1 per cent. as the total volume was 5 c.c. The figures as usual designate the number of cubic centimeters of $\frac{N}{20}$ alkali required to neutralize the acid formed.

TABLE A.

Ester.	Water.	Bile Salts.	Lecithin.	Bile Salts + Lecithin.	Bile.
Ethyl acetate	1.40	11.70	3.85	10.6	10.05
Ethyl propionate	2.55	11.30	5.60	10.55	11.35
Ethyl butyrate	2.40	6.90	6.20	9.90	6.95
Diacetin	4.95	10.85	5.95	10.20	9.55
Triacetin	9.10	14.35	11.20	15.05	13.95

The accelerations here noted are as follows:

TABLE B.—PERCENTAGE ACCELERATIONS.

Ester.	Bile Salts.	Lecithin.	Bile Salts + Lecithin.	Bile.
Ethyl acetate	736	175	657	618
Ethyl propionate	343	120	314	345
Ethyl butyrate	187	158	312	190
Diacetin	119	20	106	93
Triacetin	58	23	65	53

If we assign the value 100 to the hydrolysis of ethyl butyrate and thus determine the hydrolysis of the other esters in this series in terms of ethyl butyrate we have:

TABLE C.—ESTER VALUES IN TERMS OF ETHYL BUTYRATE.

Ester.	Water.	Bile Salts.	Lecithin.	Bile Salts + Lecithin.	Bile.
Ethyl acetate	58	170	62	107	145
Ethyl propionate	106	164	90	107	163
Ethyl butyrate	100	100	100	100	100
Diacetin	206	157	96	103	136
Triacetin	379	208	181	152	201

Table B of this series shows that with this specimen of juice the percentage acceleration was much greater with the bile salts than with lecithin. The combined effect of lecithin and bile salts was less than that noted with the bile salts alone except in the case of the ethyl butyrate. Here the effect of both (312 per cent. acceleration) was nearly equal to the sum of their effect when acting separately, *i. e.* 345 per cent. Furthermore, it will be noted that the percentage acceleration caused by the bile salts alone in almost every case closely approximates that of the bile and leads to the conclusion that in this series of experiments, at least, the accelerating action of the bile is almost entirely dependent on the content of bile salts. The bile salts used were obtained from ox bile, which contains both taurocholates and glycocholates, and since they have an activity equal to that of dog bile in which only taurocholates are present, we would conclude that the effects of taurocholates and glycocholates are about equal. It is very interesting to note in these tables (especially C) the difference between the amounts of hydrolysis which the different esters suffered. Thus when no accelerator was used the hydrolysis of the acetate was only 58 per cent. of that of the butyrate, while in the presence of the bile salts the hydrolysis of the acetate was 1.7 times that of the butyrate. Thus the effect of a given substance on the hydrolysis of one ester is no index to its effect on the hydrolysis of another ester. We wish to emphasize one point especially and that is that the results vary with different experimental conditions, such as the dilution and quantity of the juice acting, the quantity of ester used, the temperature and duration of the experiments, and finally a considerable degree of variation is seen with different specimens of juice even when the experiments are duplicated as nearly as possible. In some cases lecithin causes a greater acceleration than the bile salts. This was usually found when the juice was employed in greater dilution.

A series similar to the above was carried through with olive oil.

SERIES 8. Duration of experiments, 19 hours. Temperature, 38° C. One-half of a cubic centimeter of pancreatic juice was used in each experiment. It acted in a total volume of 5 c.c. in every case. The bile salts acted in a concentration of 2.5 per cent. both when used alone and with the lecithin. The lecithin again acted in a 0.4 per cent. suspension.

The bile was so diluted that the bile salts contained in it also acted in a concentration of 2.5 per cent. Six-tenths of a gram of olive oil was used in each experiment. The figures record the number of cubic centimeters of $\frac{N}{20}$ alcoholic KOH required to neutralize the acid produced.

	$\frac{N}{20}$ alkali required			
	(1)	(2)	Average	Per cent. of Acceleration
Water	10.85	11.60	11.22	—
Bile salts	19.25	18.25	18.75	67
Lecithin	12.3	12.5	12.4	11
Bile salts + lecithin	19.4	19.7	19.55	74
Bile	—	17.9	17.9	60

Here again we see that the bile salts in the proper concentration accelerate the action more than lecithin does and that the combined effect of lecithin and bile salts is very little greater than that of the latter when acting alone. Hammarsten[1] found the lecithin content of dog's bile as discharged from a fistula to be about 0.12 per cent., hence in our experiments probably more lecithin was present per cubic centimeter of pancreatic juice than there is normally in the intestine. We have studied the action of pancreatic juice on lecithin under the conditions of our experiments. The increase in acidity noted was negligible and was not appreciably increased when bile salts were present. Schumoff-Simanowski and Sieber[2] have recently found that pancreatic juice actively hydrolyzes lecithin. In their experiments much larger quantities of lecithin were employed than in our work. In our work the increase in acidity due to the hydrolysis of lecithin was certainly not sufficiently great to materially vitiate the results.

CONCLUSIONS.

(1) Bile salts, lecithin, and bile greatly accelerate the action of pancreatic juice on all of the esters studied including olive oil.

(2) The optimum concentration for the bile salts when the

[1] *Jahresber. über d. Fortsch. d. Thierchem.*, xxiii, p. 331, 1893. Hoppe-Seyler, *Lehrbuch der physiol. Chem.*, Berlin, 1881, p. 302.

[2] *Zeitschr. f. physiol. Chem.*, xlix, p. 50, 1906.

lower esters are used is about 0.1 per cent., while for olive oil the the optimum is from 2 to 4 per cent. In the latter concentration the bile salts greatly inhibit the action on triacetin, and the acceleration of the hydrolysis of ethyl butyrate is much less than when they are employed in greater dilution.

(3) The combined effect of the bile salts and lecithin on the hydrolysis of the esters is usually but very little greater than that of the bile salts alone. Some notable exceptions to this were observed, however. Thus in some cases the acceleration was much greater when both were used, while in other cases the acceleration was quite a little less than when the bile salts were used alone.

(4) The effect of these accelerators on the hydrolysis of one ester is no index to the degree of acceleration which will be noted when other esters are used. One cannot predict what the acceleration will be even in an homologous series of esters.

(5) Different specimens of juice and different experimental conditions altered greatly the degree of acceleration observed and the relative activity of the bile salts and lecithin.

(6) The effects of bile salts and bile of a corresponding concentration of bile salts often very closely approximate one another, and we believe the bile salts are mainly responsible for the accelerating effect of bile on the hydrolysis of fats and esters by pancreatic juice.

(7) We advance no theory to account for the acceleration noted with these substances. While we believe that their action depends to a certain extent on their solvent action, it seems that in addition to this they accelerate the action of the enzyme in some other way.

ARE THE ANIMAL ENZYMES CONCERNED IN THE HYDROLYSIS OF VARIOUS ESTERS IDENTICAL?

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(Received for publication, November 13, 1906.)

The action of extracts of animal tissues on various esters has been the subject of numerous communications in the last few years. The technical difficulties in dealing with the fats and higher fatty acids led to the introduction of simpler esters in investigations on the action of the fat-splitting enzyme. Berthelot[1] thus introduced monobutyrin which is quite soluble in water and is hydrolyzed much more readily by extracts of plant and animal tissues than the higher fats. Hanriot[2] also adopted monobutyrin in his numerous and important investigations on the subject. For similar reasons Kastle and Loevenhart[3] introduced the use of ethyl butyrate which has the great advantage of being on the market and of being readily obtained in pure condition. In 1898 Hanriot[4] compared the action of lipase (apparently serum lipase) on the ethyl esters of formic, acetic, propionic, and isobutyric acids. He found that as the molecular weight of the combined acid increases the hydrolysis by lipase decreases. In 1900 Kastle and Loevenhart[5] compared the action of pancreas extracts on the ethyl esters of formic, acetic, propionic, and n-butyric acids and arrived at results at variance with those of Hanriot. They found that in the series studied the higher the molecular weight of the combined acid the more readily the hydrolysis by pancreas extract occurs. The reverse of this holds, however, for acid hydrolysis as both acetic and hydro-

[1] Cited by Gamgee, *A Text-book of the Physiological Chemistry of the Animal Body*, London, 1893, ii, p. 212.
[2] *Compt. rend. de la soc. de biol.*, 1896–1903.
[3] *Amer. Chem. Journ.*, xxiv, p. 491, 1900.
[4] *Arch. de physiol.*, x, p. 797, 1898.
[5] *Loc. cit.*

chloric acid hydrolyzed ethyl acetate much more readily than ethyl butyrate under the conditions of our work. Chamoz and Doyon[1] studied the action of lipase on amyl salicylate and found that liver extract can hydrolyze this ester whereas pancreas extract and blood serum cannot hydrolyze it to any extent. Kastle[2] showed that the salts of the acid esters of the dibasic acids, *e. g.*, sodium ethyl succinate, are not hydrolyzed by lipase and made the suggestion that lipase and perhaps enzymes in general are incapable of acting on ionized substances. Doyon and Morel[3] studied the hydrolysis of the following esters by blood serum: the ethyl esters of acetic, propionic, butyric, valeric, caproic, succinic, benzoic, and salicylic acids, mono-, di-, and tributyrin, triacetin, amyl salicylate, and phenetol. They found that the esters of the aromatic acid are only hydrolyzed to a very slight extent while phenetol is not hydrolyzed at all. Their description of these experiments is very meagre. Dakin[4] studied the hydrolysis of the esters of mandelic acid by lipase with special reference to the relation of optical activity to the action of the enzyme. Quite recently Kastle[5] has studied the relation of the constitution of esters to lipolytic action. He found that varying the alkyl combined with lower fatty acid exerts comparatively little influence on the rate of hydrolysis by lipase, whereas varying the acid combined with a given alcohol causes great variations in the stability of the resulting esters towards lipase. Using clear solutions of liver lipase and solutions of the esters he was able to confirm the earlier observations of Kastle and Loevenhart on pancreas extract that with increasing molecular weight of the combined acid the esters show an increasing instability towards lipase. Kastle also studied the effect of introducing halogens and cyanogen into the acid radicals.

In 1900 the idea occurred to the writer that if it could be shown that lipase is reversible in its action a simple and satisfactory explanation would be offered for the mechanism by which fat is

[1] *Journ. de physiol. et de path. gén.*, ii, p. 695, 1900.
[2] *Amer. Chem. Journ.*, xxvii, p. 481, 1902.
[3] *Compt. rend. de la soc. de biol.*, liii, p. 682, 1903.
[4] *Journ. of Physiol.*, xxx, p. 253, 1904; xxxii, p. 199, 1905.
[5] Bulletin No. 26, Hygienic Laboratory, U. S. Public Health and Marine Hospital Service, p. 43, 1906.

absorbed, translocated, and deposited by the organism. Kastle and Loevenhart[1] succeeded in showing that pancreatic lipase is capable of effecting the synthesis of ethyl butyrate from ethyl alchohol and butyric acid. They held that this was equivalent to proving that the action of lipase is reversible on the ordinary fats, since in their general chemical character these substances are identical. Several weeks later Hanriot[2] stated that lipase is reversible in its action on monobutyrin. In 1902 Loevenhart[3] published further observations on the part played by lipase in fat absorption and metabolism in which the inference was again made that the work on ethyl butyrate is directly applicable to the natural fats. It has since been shown that lipase is reversible in its action on the natural and higher fats by Pottevin,[4] using pancreas lipase, and by Taylor[5] and also Dunlap[6], using the lipase of the castor bean. These facts seemed to justify the application of the results with ethyl butyrate to fat metabolism. In France, the views of Hanriot, which were reached by the use of monobutyrin and closely resemble those of Loevenhart, were opposed by Arthus in a very bitter controversy. Arthus[7] maintained that the hydrolysis of monobutyrin has nothing to do with the hydrolysis of the fats. He contended that serum which readily hydrolyzes monobutyrin is incapable of hydrolyzing the higher fats. Arthus therefore suggested that the enzyme concerned in Hanriot's experiments be called monobutyrase.

In his last publication Taylor[8] makes the following remark: "The statement of Arthus that animal lipase is a monobutyrase is a verbal quibble." To the writer's knowledge Arthus never made such a statement and this is far from his meaning. Arthus believes that *blood serum* is incapable of acting on the higher fats,

[1] *Loc. cit.*
[2] *Compt. rend. de la soc. de biol.*, liii, p. 70, 1901.
[3] *Amer. Journ. of Physiol.*, vi, p. 331, 1902.
[4] *Compt. rend. de l'Acad. des sci.*, cxxxvi, p. 1152, 1903; cxxxviii, p. 378, 1904.
[5] *Univ. of Cal. Pub., Path.*, i, p. 33, 1904.
[6] Private communication.
[7] *Journ. de physiol. et de path. gén.*, iv, p. 56, 1902.
[8] This Journal, ii, p. 87, 1906.

although it possesses marked activity toward monobutyrin. Hence Arthus objects to the term lipase being applied to the enzyme of the *serum* which according to him does not split the fats. He therefore suggested that the enzyme of the *serum* be called monobutyrase. He made no reference to animal lipase in general. Connstein[1] has attempted to criticise the results and conclusions of Hanriot but his remarks are so pointless and biased that they demand but passing notice. Thus he endeavors to show that the hydrolysis of monobutyrin by blood serum is not to be attributed to an enzyme at all, but to the proteids of the serum and states that he has unpublished results which bear this out. In the two years which have elapsed, these results have unfortunately remained unpublished so far as the writer has been able to find. This view is absolutely unwarranted. There can be no doubt that the hydrolysis of monobutyrin by serum and extracts of most living tissues must be included in the group of reactions called fermentative or enzymic, and the only thing which is open to question is whether we are to attribute the hydrolysis of the esters of the lower fatty acids and the fats to one and the same enzyme or to different enzymes. The fats and the esters of the lower fatty acids are hydrolyzed and synthesized by the same chemical methods and are absolutely analogous from a chemical point of view. It is true that they differ widely in their physical properties, but are these of such character as to necessitate entirely different enzymes in these processes? Then, if we assume that different enzymes are required for the hydrolysis of fats and lower esters, the question naturally arises whether the hydrolysis of each ester would not have to be regarded as a distinct activity and attributed to a distinct enzyme. Such an hypothesis could only be accepted on the strongest evidence, as the action of enzymes is not at present regarded as so specific as this. The esters with which we are dealing present no possibilities for optical isomerism which might account for specificity of action. In the present state of knowledge, it would be far more logical to assume a special pepsin or trypsin for each proteid, because in this group comparatively little is known of the structure,

[1] *Ergebnisse der Physiologie*, iii, 1, p. 194, 1904.

and the possibilities of isomerism, optical or otherwise, are enormous.

Hanriot[1] working with monobutyrin concluded that the enzyme of blood serum which hydrolyzes this ester is different from that contained in pancreas extract. He drew this conclusion from two facts: (1) He made preparations of serum and pancreas which had the same activity in the presence of sodium carbonate and found that when they were exactly neutralized the serum preparation was nearly twice as active as that of the pancreas. (2) Preparations of serum and pancreas were made of the same activity at 15° C. and he found that at 42° C. the serum preparation became twice as active as that of the pancreas. Kastle and Loevenhart[2] found that similar differences could be made out between liver and pancreas preparations acting on ethyl butyrate. They observed that here also the preparations react differently to changes of temperature and further that certain substances affect the two preparations differently. Using preparations of liver and pancreas which hydrolyze equal amounts of ethyl butyrate in experiments of short duration, Loevenhart[3] found that if allowed to act until equilibrium is reached the hydrolysis with the liver preparation is much more complete than in the case of the pancreas preparation. These comprise the only efforts, so far as the writer is aware, to throw light on the subject under consideration.

We have endeavored to determine the identity or non-identity of these enzymes by studying (1) The relative activity of various preparations of the liver and pancreas of different animals on several esters in order to see whether there is a correspondence between these activities. (2) The effect of adding various substances in order to determine whether all of these processes are equally affected under similar conditions. (3) The effect of heating at various temperatures the pancreas powders which are described below, in order to determine whether the activity toward certain esters is impaired more than toward other esters.

[1] *Compt. rend. de la soc. biol.*, xlix, p. 377, 1897.
[2] *Loc. cit.*
[3] *Loc. cit.*

EXPERIMENTAL METHODS.

The sources of our enzymic preparations were the dog, pig, and beef. The following preparations of the enzyme were employed:

(1) 10 per cent. turbid extracts of the organs already described.[1]

(2) Clear 10 per cent. extracts of the liver already described.[2]

(3) Pancreatic juice. The results with the juice are presented in the previous communication.[3]

(4) Dry powders almost free of fat which have preserved their activity practically unaltered for eighteen months were prepared from the pancreas and liver of the dog, pig, and beef by the following method:

Ten per cent. extracts were made by grinding ten parts of freshly removed tissue with coarse white sand and extracting with a hundred parts of water. The extract was then strained through cloth. Twenty c.c of a saturated solution of uranyl acetate in distilled water were then added per 100 c.c. of extract used. This mixture was usually acid and was made neutral to litmus by adding a few drops of a solution saturated with both sodium carbonate and sodium phosphate. Only a few drops of this were required per 100 c.c. Then 5 c.c. of a saturated solution of sodium phosphate were added per 100 c.c. of extract used. The precipitate was then separated by filtering or by centrifuging and then placing on a filter to drain. The precipitate was then air-dried, several days being required to get it entirely dry. As long as the precipitate remains moist it must be sprinkled with toluene occasionally to prevent putrefaction and the growth of moulds. After air-drying the material was removed from the paper and pulverized in a mortar. The powder was then extracted for several hours with ether in a Soxhlet extractor, again ground up and passed through the finest sieve, and air-dried. The resulting powder was always active although the activity varied somewhat in different preparations.

The above method resembles Rosell's[4] modification of Jacoby's method but differs from it in that instead of having the enzyme in solution, we have it in the form of an insoluble fat-free powder. The method is of interest in so far as it proves the resistance

[1] This Volume, p. 397.

[2] *Ibid.*, p. 398.

[3] *Ibid.*, p. 415.

[4] Inaug. Diss., Strassburg, 1901. Cited by Magnus, *Zeitschr. f. physiol. Chem.*, xlii, p. 151, 1904.

of animal lipase to the prolonged action of ether. In fact the preparations seemed to improve in activity by the treatment with ether. It has frequently been noted that methods of obtaining preparations of lipase which involve the use of alcohol are in general very unsatisfactory because the resulting preparations are usually only very slightly active. Taylor[1] has shown that the lipase of the castor bean is not injured by prolonged treatment with ether and by this means he prepared a similar insoluble powder from it which was very active.

The following data will give an idea of the activity of our preparations:

One gram of pancreas powder was ground up with 50 c.c. of distilled water so that as homogeneous a suspension as possible was obtained. Five c.c. of this suspension were treated with 0.26 c.c. ethyl butyrate and after standing 15 minutes at 40° C. the amount of acid produced by the action of the enzyme on the ester was determined by titration with $\frac{N}{20}$ sodium hydroxide using litmus as the indicator. 2.85 c.c. of sodium hydroxide were required, corresponding to 7.12 per cent. decomposition. A similar experiment performed with a suspension of liver powder after acting 15 minutes at 40° C. required 1.05 c.c. of sodium hydroxide which corresponds to 2.62 per cent. decomposition.

The liver powders were invariably found to be less active than the pancreas powders. This was somewhat surprising since Kastle and Loevenhart found that the extract of pig liver is from two to three times as active on ethyl butyrate as the pancreatic extract. In order to determine what per cent. of the enzyme passes into solution on grinding the powders up with water, portions of the above-described suspensions were centrifugalized and the activity of the turbid supernatant fluid determined as above. The pancreas required 1.45 c.c. of sodium hydroxide, viz., 3.62 per cent. decomposition. The liver suspension required 0.35 c.c. of sodium hydroxide, viz., 0.87 per cent. decomposition. Thus merely by centrifuging the pancreas suspension lost 49 per cent. of its activity and the liver lost 67 per cent. The pancreas powders contain some of the other enzymes of the pancreas, namely, trypsin and diastase, but no rennin action could be made out. The diastatic action of the powder was quite weak.

[1] *Univ. of Cal. Pub., Path.*, i, p. 33, 1904.

The esters used and the quantities in which they were employed are as follows: Diacetin—0.1714 gram, triacetin—0.1497 gram, ethyl acetate—0.1747 gram, ethyl propionate—0.2082 gram, ethyl butyrate—0.23 gram, methyl caprylate—0.316 gram, methyl laurate—0.425 gram, methyl palmitate—0.3406 gram, and neutral olive oil—0.3 gram. All of these esters had been fractionated and were pure. The methyl esters of caprylic, lauric, and palmitic acids were furnished by Dr. F. L. Dunlap of Ann Arbor. I wish here to express my thanks to him for his kindness in letting me have them. He had fractionated *in vacuo* the esters prepared from Kahlbaum's C. P. acids. The quantities of the esters used in all cases, except with the palmitate and olive oil, would require approximately 40 c.c. of $\frac{N}{20}$ alkali for complete saponification.[1] The quantity of methyl palmitate used (0.3406 gram) would require 25.23 c.c. of $\frac{N}{20}$ alkali, while the olive oil (0.3 gram) would require 21.36 c.c. $\frac{N}{20}$ alkali for complete saponification. With these data it is easy to calculate the per cent. hydrolysis in any case. In some experiments aqueous solutions of the esters were employed, but these will be described in connection with the experiments.

The titrations were made with $\frac{N}{20}$ aqueous sodium hydroxide in the experiments with the lower esters, and $\frac{N}{20}$ alcoholic potassium hydroxide[2] in the experiments with the higher esters. Litmus or phenolphthalein was used in the aqueous titration and the latter in the alcoholic ones. The numbers given in the tables unless otherwise stated give the amount of $\frac{N}{20}$ alkali in c.c. required to neutralize the acid formed. The initial acidities and blanks have been taken into account and the numbers given represent the increase in acidity due to the action of the enzyme on the ester. The duration and temperature will be given in connection with each series.

THE RELATIVE ACTION OF LIVER AND PANCREAS EXTRACTS ON VARIOUS ESTERS.

We have not succeeded in obtaining clear pancreas extracts

[1] The reasons for using the esters in acid equivalent quantities are given on page 398 of this volume.

[2] When alcoholic KOH was used, neutralized 85 per cent. alcohol was always added as described on p. 399 of this volume.

comparable in activity to that of the turbid extracts. Hence only turbid extracts of liver and pancreas have been used in this part of the work. Neglecting for the present the possible effect of admixtures present in the extracts with the enzyme it is obvious that if the same enzyme is present in the liver and pancreas, and if this one enzyme brings about the hydrolysis of all the esters, then whatever ratio obtains between the activity of given liver and pancreas extracts when any given ester is used should obtain also when the same extracts act upon any other ester under identical conditions. Thus if the hydrolysis of three esters by a pancreas extract be represented by 100, 60, and 40 and the action of a liver extract on the first under the same conditions be represented by 200, then we would expect the action of the liver extract on the second and third to be 120 and 80 respectively. In this case the liver-pancreas ratio would be 2 regardless of which ester was used in making the comparison. Such findings would prove that the hydrolysis of the three esters is brought about by the same enzyme. Negative results would not necessarily prove that we have here to deal with different enzymes. But this will be considered later. The liver-pancreas ratio ($\frac{L}{P}$) has been determined for a number of esters and will always be recorded in the tables which follow. As stated, however, $\frac{L}{P}$ will only be constant for different esters if the liver and pancreas contain the same enzyme which hydrolyses all of the esters used. It may be, however, that liver and pancreas each contain a single ester-splitting enzyme but that these two enzymes are different. If the liver contains a single lipolytic enzyme then we would expect to find the ratio of the activity of a given liver extract on any two esters to be the same for every other preparation of the liver under the same conditions. Thus if the activity of a liver extract on ethyl butyrate be represented by 100 and on triacetin by 50, then we should expect this ratio to hold for every other liver preparation when acting on these esters, under identical conditions. It is perfectly obvious that the preparations will vary in their absolute activity on the esters, but if one enzyme is alone accountable for these processes we would expect the same ratio always to hold between two given esters.[1] Since ethyl butyrate has been used more

[1] Since this paper was sent to press I have found that the ester values

frequently in this work than any other ester it is used as the basis of comparison, and the amount of alkali required to neutralize the acid formed in its hydrolysis has been placed in every case at 100. The amount of hydrolysis of the other esters has been computed on this basis and this is also recorded in the tables. The numbers thus obtained will be referred to as the corresponding ester value.

SERIES 1. Duration of experiments, 17 hours. Temperature, 39° C. One c.c. of a 10 per cent. extract of liver or pancreas of a dog and 4 c.c. of water were used in each experiment.

	Ethyl butyrate.	Triacetin.	Triacetin value.
Liver	8.65	5.15	59.5
Pancreas	1.80	2.70	150
$\frac{L}{P}$	4.8	1.9	

SERIES 2. Similar in all respects to Series 1, except that the extracts of the organs of another dog were used.

	Ethyl butyrate.	Triacetin.	Triacetin value.
Liver	5.15	5.35	104
Pancreas	1.2	3.2	267
$\frac{L}{P}$	4.3	1.67	

Series 1 and 2 show that $\frac{L}{P}$ is about 2.5 times as great when ethyl butyrate is used in making the comparison as when triacetin is used. On the supposition that the liver and pancreas each contain only one ester-splitting enzyme, these experiments would indicate that these two enzymes are different. Furthermore it will be noted that the triacetin values for the liver and pancreas as well as the value of the $\frac{L}{P}$ with each ester showed considerable variation in the two series. It must be emphasized that we are not interested in the absolute values in any case, as it might be expected that certain individuals would

vary to a great extent with slight alterations in the conditions of the experiments. Thus a ten per cent. turbid extract of pig's liver which has stood twenty-four hours in the incubator gives an ethyl acetate value of 110. If this extract is simply filtered it gives an ethyl acetate value of 60. Hence the turbid extract is slightly more active on the acetate than on ethyl butyrate while the same extract simply filtered is only 0.6 as active on the acetate as on the butyrate.—*Note added during proof-reading.*

yield more active preparations than others. The ratios should be the same, however, in each case if all the enzymes concerned are the same. Although considerable variation is seen in different individuals of the same species it was found on comparing the results obtained with extracts of dog, pig, and beef organs that the different species show considerably greater variation in these values. Quite a number of experiments were made in order to determine the average results for these species, and on account of variations encountered with individuals of the same species the number of experiments should have been much larger. The following is a summary of the averages obtained with the above species. The experiments were performed with 1 c.c. of 10 per cent. extract and 4 c.c. of water. Duration, 18 hours. Temperature, 39° C.

TABLE I. VALUES OF $\frac{L}{P}$

	Ethyl butyrate.	Diacetin.	Triacetin.
Dog	4.69	0.84	1.4
Pig	2.52	1.06	0.77
Beef	1.51	1.94	2.88

The ester values found in the same experiments average as follows:

ESTER VALUES.

	Diacetin	Triacetin.
Liver.		
Dog	35	64
Pig	28.5	38
Beef	178	203
Pancreas.		
Dog	213	203
Pig	71	124
Beef	134	132

This table shows that (1) $\frac{L}{P}$ varies with different esters when the same extracts are used; (2) with different species when the same ester is used; and it has also been pointed out that it varies to a considerable extent with different individuals of the same species when the same ester is used. It will be seen that on ethyl butyrate dog liver is 4.69 times as active as dog pancreas, while beef liver is only 1.51 times as active as beef pancreas. When the comparisons are made with triacetin, dog liver is 1.4 times as active as dog pancreas, while beef liver is 2.88 times as active as beef pancreas. Hence $\frac{L}{P}$ with ethyl butyrate is 3 times as large for the dog as for the beef, but with triacetin $\frac{L}{P}$ for the

beef is twice as large as for the dog. An examination of the table shows that there is no parallelism even between diacetin and triacetin. The differences between the latter esters are, however, much smaller than between them and the butyrate. This fact is also strikingly brought out in the ester values. It has been found through out this work that the nearer two esters are related the more nearly will the results obtained with them correspond. The ester values vary to a certain extent with different individuals of the same species and to a greater extent with different species.

In order to indicate further how $\frac{L}{P}$ will vary with different conditions and to show the effect of dilution, the following experiments are given.

Three dogs were starved for 2 days and killed under anæsthesia by bleeding from the portal vein. Ten per cent. extracts of the liver and pancreas were prepared in each case. The duration of all the experiments was 24 hours. Temperature, 38° C. All of these experiments were performed at the same time. In the first set of experiments 5 c.c. of the extracts were employed, while in the second set 1 c.c. of the extracts and 4 c.c. of water were used.

Series 4. First set of experiments, 5 c.c. of the extracts used.

		Ethyl butyrate.	Triacetin.	Triacetin value.
Dog 1.				
	Liver	12.3	10.31	84
	Pancreas	2.65	5.3	200
	$\frac{L}{P}$	4.64	1.95	
Dog 2.				
	Liver	11.75	14.0	119
	Pancreas	2.6	6.05	233
	$\frac{L}{P}$	4.5	2.3	
Dog 3.				
	Liver	11.0	14.0	127
	Pancreas	3.45	5.25	152
	$\frac{L}{P}$	3.2	2.7	

Second set of experiments: 1 c.c. of the same extract and 4 c.c. of water were used in each experiment.

		Ethyl butyrate.	Triacetin.	Triacetin value.
Dog 1.				
	Liver	7.15	8.15	114
	Pancreas	0.7	0.95	136
	$\frac{L}{P}$	10.2	8.6	
Dog 2.				
	Liver	6.55	7.05	106
	Pancreas	0.65	1.4	215
	$\frac{L}{P}$	10.2	5.0	

		Ethyl butyrate.	Triacetin.	Triacetin value.
Dog 3.	Liver	6.25	8.35	134
	Pancreas	0.55	1.1	200
	$\frac{L}{P}$	11.4	7.6	
Average of $\frac{L}{P}$ values.				
	Set 1	4.1	2.3	
	Set 2	10.6	7.1	
	Average result for dog p. 437	4.69	1.4	

Apart from the interesting individual variations, these experiments show (1) that the liver activity compared to that of the pancreas is much greater when the extracts are diluted; (2) that the animals killed in this way after a period of starvation present marked differences from animals killed under other conditions. The experiments from which the figures in Table 1 (p. 437) were obtained were performed with the diluted extracts and are comparable to set 2 of Series 4. It will be seen that $\frac{L}{P}$, determined with butyrate, is doubled and $\frac{L}{P}$, determined with triacetin, is increased five times in these dogs. The triacetin values with the starved dogs were much larger with the liver than the other animals studied, while the triacetin values with the pancreas were about the same as with ordinary dogs. These animals seemed to differ principally in the great activity of the liver. Dilution affected the triacetin values only very slightly. These facts can be readily seen in the following summary:

TRIACETIN VALUES FOR STARVED DOGS.

		(1)	(2)	(3)	Average.
Set 1.	Liver	84	119	127	110
	Pancreas	200	230	152	194
Set 2.	Liver	114	106	134	118
	Pancreas	136	215	200	184
				Liver.	Pancreas.
Average triacetin value for both sets (starved dogs)				114	189
Average triacetin value for fed dogs, p. 437				64	203

Similarly the activities of liver and pancreas extracts of the dog, pig, and beef on olive oil have been compared. The results obtained were quite different from those with the lower esters. The following experiments give an idea of the activity of these extracts on olive oil.

SERIES 5. Ten per cent. extracts of the liver and pancreas of a dog were prepared in the usual way.

(1) 10 c.c. liver extract.
0.3 gm. olive oil.
0.2 c.c. toluene.

(2) 10 c.c. pancreas extract.
0.3 gm. olive oil.
0.2 c.c. toluene.

The flasks were corked and well shaken at the beginning of the experiment. They were placed in the thermostat at 38° C. After 17 hours they were titrated with alcoholic KOH as described above.

(1) required	5.3 c.c.	$\frac{N}{20}$ KOH	
	3.2 c.c.	" "	initial acidity
	2.1 c.c.	" "	increase in acidity.
(2) required	25.6 c.c.	$\frac{N}{20}$ KOH	
	4.7 c.c.	" "	initial acidity.
	20.9 c.c.	" "	increase in acidity.

Here the liver-pancreas ratio is 0.1. As the result of a large number of experiments with extracts of the liver and pancreas of the dog, pig, and beef the average liver-pancreas ratio for olive oil was 0.134. Therefore the relative activity of extracts of the liver and pancreas when acting on the esters of the lower fatty acids is quite the reverse of that found when their activity on the higher fats is studied. Unless this striking fact can be explained we must assume that the liver and pancreas contain different enzymes or attribute the hydrolysis of the lower esters to a different ferment from that which effects the hydrolysis of the higher fats.

The effect of mixing extracts of the pancreas and liver on the rate of hydrolysis of the lower esters and fats was investigated in the hope of throwing further light on these facts. It has been shown that the bile salts act as specific accelerators in the hydrolysis of amyl salicylate by Magnus' clear liver extracts, and it has also been pointed out that sodium fluoride inhibits the action of pancreas on esters far more than on fats. Now the fact that the liver is more active on the esters and the pancreas more active on fats could be explained in one of two ways without assuming the liver and pancreas contain different enzymes or that the hydrolysis of the fats and esters of the lower fatty acids are to be attributed to different enzymes. (1) The liver might contain a more or less specific inhibitor for the action of the enzyme on the fats. (2) The pancreas might contain a more or less specific accelerator for the action of the enzyme on fats. In the former case we should expect to find that on mixing liver extract with that of the pancreas the activity of the pancreas on olive oil

would be diminished. In the latter case we should expect to find that mixing liver and pancreas extracts would cause a marked acceleration of the hydrolysis of olive oil, because the accelerator present in the pancreas would render available all of the lipase present in the liver. The results of the experiments on mixtures of the extracts acting on the lower esters and olive oil follow;

SERIES 6. Duration of experiments, 15 minutes. Temperature, 40° C. Ten per cent. extracts of the liver and pancreas of a dog were employed.

(1)	(2)	(3)
2.0 c.c. pancreas ext.	2.0 c.c. liver ext.	2.0 c.c. pancreas
4.0 " water	4.0 " water	2.0 " liver ext.
0.26 " ethyl butyrate	0.26 " butyrate	2.0 " water
0.1 " toluene	0.1 " toluene	0.26 " butyrate
		0.1 " toluene

(1) required 1.7 c.c. $\frac{N}{20}$ NaOH
(2) " 2.85 " " "
(3) " 4.5 " " "
Sum of (1) and (2), 4.55 " " "

Similar results were obtained when triacetin was used.

Hence mixtures of liver and pancreas extracts when acting on the esters of the lower fatty acids show merely the activity of the ingredients of the mixture, that is, no acceleration or retardation is noted.[1] When olive oil is used, on the other hand, a remarkable acceleration is noted, as shown in the following experiments.

SERIES 7. Duration of experiment, 16 hours. Temperature, 37° C. Ten per cent. extracts of pig's liver and pancreas used.

No.	5 c.c. Pancreas Ext.	5 c.c. Liver Ext.	5 c.c. Water.	5 c.c. Boiled Pancreas Ext.	5 c.c. Boiled Liver Ext.	0.3 gm. Olive Oil.	Increase in Acidity	Per cent of Acceleration.
1	+		+				7.30	
2	+		+			+	10.65	
3			+	+		+	0.00	
4		+	+				0.8	
5		+	+			+	1.55	
6			+		+	+	0.00	
7	+	+					16.95	109
8	+	+				+	26.4	116
9	+				+	+	9.55	
10		+		+		+	2.95	90

[1] In experiments with triacetin some retardation is usually noted.

From Series 7 it will be seen that by mixing extracts of the liver and pancreas the action on olive oil is accelerated to double the activity of the extracts when acting separately. The increase in acidity in 1, 4, and 7 in which no olive oil was present is undoubtedly principally due to the hydrolysis of the fat contained in the extracts, as it is impossible to prepare an extract, of the pancreas specially, which is not very rich in fat. That the increase in the acidity is due to the hydrolysis of fat is indicated by the fact that the addition of olive oil greatly increases the production of acid, although the per cent. acceleration noted with mixtures of liver and pancreas was practically the same whether olive oil was added or not. Boiled liver extract in this series (Exp. 9) caused no acceleration in the action of pancreas extract; in fact a slight retardation is noted. In other cases boiled liver extract caused some acceleration, but this was never comparable with that produced by fresh liver extract. The boiled pancreas extract accelerated the action of the liver extract 90 per cent., but the action of the liver is so weak that this is entirely insufficient to explain the increased action noted when both extracts are employed in the active state. In order to determine the most favorable mixture of the extracts to show the acceleration the following experiments were tried:

SERIES 8. Duration of experiments, 24 hours. Temperature, 38° C. Ten per cent. extracts of pig liver and pancreas. 0.3 gm. of olive oil was used in each experiment.

No.	Pancreas Ext. c.c.	Liver Ext. c.c.	Water c.c.	$\frac{N}{20}$ KOH Required	Per cent. of Acceleration
1	0.5		9.5	3.41	
2	2.5		7.5	7.21	
3	5.0		5.0	9.97	
4	10.0		.	21.6	
5		10.0		2.55	
6	0.5	9.5		8.34	43
7	1.0	9.0		16.33	138*
8	2.5	7.5		20.60	126

*As no corresponding experiment with 1 c.c. pancreas extract + 9 c.c. water was made in this series the hydrolysis by 1 c.c. pancreas extract +9 c.c. of water was estimated from Experiments 1 and 2 to be 4.56.

In this series (8) of experiments the greatest acceleration is seen in Experiment 7, in which 1 c.c. of pancreas extract and 9 c.c. of liver extract were employed. These quantities of the extracts when acting separately produced an amount of acid corresponding to 6.85 c.c. of $\frac{N}{20}$ potassuim hydroxide, but when acting together required 16.33 c.c. These results proved clearly that the liver does not contain a substance which inhibits the hydrolysis of olive oil by the enzyme, and they were at first interpreted as indicating that the pancreas containsanaccelerator, specific for the action of the enzyme on fats. This substance would play a part in this reaction analogous to that played by the bile salts in the hydrolysis of amyl salicylate by the clear liver extracts. It was proved, however, that the power of the liver to accelerate the action of the pancreas on olive oil is in nowise connected with its power to hydrolyze the esters of the lower fatty acids. This proof lay in the fact that the extracts of other organs which have less than one tenth the activity on ethyl butyrate that the liver has, are capable of accelerating the action of the pancreas extracts on olive oil to the same extent that the liver extract does. Thus in a series of experiments it was found than an extract of the testicle, which had only one ninth the activity of the liver extract on ethyl butyrate, accelerated the hydrolysis of olive oil by pancreas extract to an extent equal to that of the liver extract. Hence the idea of a specific accelerator in the pancreas had to be given up.

Many experiments were performed with such mixtures as liver and spleen, and liver and testicle, but no accelerations were noted except when pancreas extract was used as one of the ingredients of the mixture. It was not determined whether the accelerations noted on mixing pancreas extract with that of other organs are due to the latter accelerating the lipase contained in the former, or whether the reverse is the case, since boiling either ingredient greatly diminishes the action of the mixture. The results prove conclusively, however, that the acceleration noted with mixtures of liver and pancreas is not related to the power of the liver to hydrolyze the esters of the lower fatty acids.

The work on the mixtures of organ extracts, instead of explaining the lack of parallelism between the hydrolysis of the higher fats and the esters of the lower fatty acids, and enabling us to attribute

the hydrolysis of all to a single ferment, brought out a further and striking difference between them, namely, that by mixing an extract of the pancreas with that of certain other organs the action on the fats is remarkably acclerated, while the action on the esters of the lower fatty acids is merely that of the ingredients of the mixture. These experiments taken alone would indicate that the enzymes which hydrolyze the lower esters and the fats are different.

The average value of $\frac{L}{P}$ determined with organs of the pig on ethyl butyrate was 2.52. The same determined with olive oil was 0.14. It seemed to the writer to be of interest to determine whether the value, $\frac{L}{P}$ gradually decreases as higher esters are used, or whether it increases up to a certain ester in the fatty-acid series and then suddenly drops to the low value obtained with olive oil. Accordingly determinations were made with all the esters of the fatty-acid series obtainable. Ten per cent. extracts of pig liver and pancreas were employed; 1 c.c. of the extract and 4 c.c. of water were treated with equivalent quantities of the esters and allowed to act for 16 hours at 39.° The results were as follows:

TABLE II.

Ester.	C.C. of $\frac{N}{20}$ Alkali Required.		$\frac{L}{P}$	Ester Values.	
	Liver	Pancreas		Liver.	Pancreas.
Ethyl acetate	14.15	1.3	10.9	108	32
" propionate	14.75	2.15	6.9	112	53
" butyrate	13.15	4.07	3.2	100	100
Methyl caprylate	22.43	18.25	1.23	166	634
" laurate	3.05	7.48	0.42	23	260
" palmitate	0.52	1.88	0.28	4	46
Olive oil[1]	1.55	10.65	0.14	12	262
Diacetin	3.75	2.9	1.29	28.5	71
Triacetin	5.00	5.04	1,00	38.0	124

This series shows a gradual decrease in the value, $\frac{L}{P}$, as the esters of the higher fatty acids are approached—that is, the liver is relatively less, and the pancreas relatively more, active.

[1] The values for the esters below the line were the average results determined with extracts from other pigs.

Among the esters studied methyl caprylate is the most readily hydrolysed both by liver and pancreas extracts. On this ester the liver extract is still more active than the pancreas and the value, $\frac{L}{P}$, drops below 1, somewhere between the esters of caprylic and lauric acids. The methyl esters of caprylic and lauric acids were used because the ethyl esters were not obtainable. Kastle[1] has shown however that with the lower fatty acids very little difference toward lipase is shown by corresponding methyl and ethyl esters. Hence very similar figures would probably have been obtained had the ethyl esters been used.

Table II also shows that the glycerine esters of acetic acid are much less readily hydrolyzed by liver extract than ethyl acetate, while the reverse holds true for the pancreas. The latter hydrolyzes triacetin and, in most cases also, diacetin more readily than ethyl acetate. This effect of the alcohol is of much interest. It is very probable that the glycerides of the higher fatty acids are more readily hydrolyzed by pancreas than the methyl and ethyl esters.

Before leaving this part of the work the results of Loevenhart and Souder with pancreatic juice should be recalled. They obtained the following results when pancreatic juice of the dog was allowed to act on the series of esters:

Ester	$\frac{N}{20}$ NaOH Required.	Ester Values.
Ethyl acetate	1.4	58
" propionate	2.55	106
" butyrate	2.4	100
Diacetin	4.95	206
Triacetin	9.10	379

It will be observed on comparing these results with Table II that the results obtained with the juice are quite different from those obtained with either the liver or pancreas extracts. Thus the activity of the juice toward diacetin and triacetin resembles that of the pancreas extract more than that of the liver, but on these esters the juice is relatively much more active than either of the extracts. The ester values obtained with the juice resemble more nearly those obtained with the extracts of dog pancreas. (See Table ii., p. 437.)

[1] Bulletin No. 26, Hygienic Laboratory of the U. S. Public Health and Marine Hospital Service, 1906, p. 43.

The conclusions to be drawn from this part of the work are as follows:

(1) The activity of a preparation on any ester may vary more or less independently of the activity toward any other ester.

(2) In an homologous series of esters there is seen a more or less regular change in the reactivity toward given preparations. Thus the value of $\frac{L}{P}$ gradually falls from 10.9 with ethyl acetate to 0.28 with methyl palmitate in the series here presented. The greater the differences between two esters the greater will be the differences in the results obtained with them. This is a very striking fact. If the hydrolysis of each ester were due to a separate enzyme, no reason for this regularity would be assignable as far as the writer can see.

(3) Marked differences are observable between ethyl acetate on the one hand and diacetin and triacetin on the other. Ethyl acetate is much more readily hydrolyzed by liver extract, while the acetins are more readily hydrolyzed by pancreas extract and especially by pancreatic juice. Triacetin is more readily hydrolyzed by all of the preparations than diacetin, but even between these two esters no parallelism exists. This is the more interesting because both of these esters are freely soluble in water and the products of their hydrolysis are identical. Triglycerides in general may be more readily hydrolyzed and synthesized than diglycerides.

THE EFFECT OF CERTAIN SUBSTANCES ON THE HYDROLYSIS OF VARIOUS ESTERS

Sodium fluoride.—Loevenhart and Peirce[1] found the hydrolysis of all the esters investigated to be greatly inhibited by this substance. Great quantitative differences are to be noted, however, with different esters. Thus the hydrolysis of ethyl butyrate is from 100 to 1000 times as sensitive to the action of the fluoride as the hydrolysis of olive oil, and the hydrolysis of the esters of acetic acid is about 10 times as sensitive as that of ethyl butyrate. In extremely dilute solutions, varying with different esters, sodium fluoride may accelerate all of these processes. Hence there is a striking qualitative similarity in the

[1] This volume, p. 397.

effect of sodium fluoride on all of these processes and equally remarkable quantitative differences. Here again as we pass from the lower to the higher esters of the fatty acid series more fluoride is required to bring out corresponding effects. Liver extract is more sensitive to the fluoride than pancreas extract.

The Bile Salts.—Loevenhart and Souder[1] have discussed the effect of the bile salts on the activity of pancreatic juice. They found that the bile salts accelerate the action of the juice on all the esters studied, but here again different esters require different concentrations of the bile salts. Thus concentrations of bile salts required to accelerate the action on olive oil greatly inhibit the action of pancreatic juice on triacetin. The tables on page 422 show the effect of bile salts (0.1 per cent.) on the hydrolysis of a series of esters. It will be seen that the bile salts cause a different degree of acceleration with each ester. A few experiments were performed to determine the effect of the bile salts on the action of pancreas extract.

SERIES 9. Duration of experiments, 16 hours. Temperature, 38° C. Five c.c. of a turbid extract of pig's pancreas were used in each experiment, together with 5 c.c. of a 1 per cent. solution of the bile salts of 5 c.c. of water.

Ester.	Water.	Bile Salts.	$\frac{N}{20}$ Alkali Required. c.c.	Per cent. of Acceleration.
Ethyl butyrate	+	—	13.35	—
" "	—	+	25.05	88
Triacetin	+	—	15.45	—
"	—	+	18.15	17
Olive oil	+	—	12.75	—
" "	—	+	13.90	9

These experiments merely prove that the bile salts can accelerate the action of pancreas extract on the esters in general. The effect of bile salts on the action of pancreatic extract is quite variable and in many instances the accelerations noted are very slight. Here again the concentration of the bile salts is of

[1] This volume, p. 415.

importance. In the following series of experiments the conditions were accidentally found in which the bile salts accelerated the action on diacetin and retarded the action on triacetin.

SERIES 10. Duration of experiments, 21 hours. Temperature, 39° C. One c.c. of 10 per cent. pancreas extract, 4 c.c. of water and 1 c.c. of water or 1 c.c. of a 1 per cent. solution of bile salts were used in each experiment.

Ester.	Water.	Bile Salts.	C.C. of $\frac{N}{20}$ NaOH Required.
Diacetin	+	—	5.20
"	—	+	8.25 (Acceleration 59%)
Triacetin	+	—	18.10
"	—	+	11.55 (Inhibition 93%)

It is certainly remarkable to see such differences between two esters so closely allied. It is the most notable instance of the independence of these activities found in this work. It is to be noted that pancreas extract without any addition is far more active on most of the esters than pancreatic juice. This may be due in part to the lecithin content of the pancreas.

The effect of the bile salts on liver extract is very interesting. As already pointed out they are essential in the hydrolysis of amyl salicylate by clear liver extract. The bile salts do not increase the hydrolysis of olive oil by turbid liver extracts. The bile salts usually inhibit the action of turbid and clear liver extracts on the esters of the lower fatty acids. For instance, the bile salts acting in a concentration of 0.5 per cent. inhibited the action of turbid liver extract on ethyl butyrate 91 per cent. and on triacetin 85 per cent. Very interesting results were obtained in the experiments with clear 10 per cent. liver extract. In these experiments aqueous solutions of ethyl acetate, propionate and butyrate were used, and as the experiments were of short duration no toluene was used. These experiments were therefore conducted in water-clear solutions.

SERIES 11. Duration of experiments, 2 hours. Temperature, 30° C. The amounts of the ester solutions employed in each experiment were equivalent to 10 c.c. of a $\frac{N}{20}$ solution and the total volume in every case was made up to 15.4 c.c. with the extract and water or solutions of the

bile salts. One c.c. of 10 per cent. clear liver extract was used in every experiment. The figures express the percentage of the ester hydrolyzed.

Ester.	Water.	Bile Salts. 0.33 per cent.	0 67 per cent.	1 per cent.
Ethyl acetate	14.1	2.5	3.7	5.2
" propionate	46.0	5.6	6.8	11.2
" butyrate	69.7	5.0	7.2	11.2

The percentage inhibition caused by the bile salts in this series was as follows:

Ester.	Bile Salts. 0.33 per cent.	0 67 per cent.	1 per cent.
Ethyl acetate	82	74	63
" propionate	88	85	76
" butyrate	93	90	84

It will be seen that the hydrolysis of the propionate was in most case intermediate between that of the acetate and butyrate. The percentage inhibition in the case of the propionate is almost a mean between that of the acetate and butyrate. Kastle[1] found that in the hydrolysis of these esters in similar experiments the propionate approaches a mean between the other two. He did not study the effect of the bile salts on the reaction. It will also be noted that the inhibiting action of the bile salts increases with the molecular weight of the acid of the ester and this explains why the hydrolysis of the propionate may exceed that of the butyrate in the presence of the bile salts. In this respect the effect of the bile salts is quite the opposite of that of sodium fluoride. It is also striking that the inhibiting effect of the bile salts is much greater in the more dilute solution, 0.33 per cent., than in greater concentrations, 0.67 per cent. and 1 per cent.; and in this connection a most interesting parallelism was found to exist between the inhibition noted and the degree of turbidity which always developed in the experiments with the bile salts. In the experiments without the bile salts the solutions remained perfectly clear. All those which contained bile salts became turbid,

[1] Bulletin No. 26, Hygienic Laboratory of the U. S. Public Health and Marine Hospital Service, p. 43, 1906.

and the greater the turbidity developed the greater was the inhibition noted. Thus the turbidity was greatest in the flasks with the butyrate and least in the acetate experiments. Moreover the turbidity was greatest in the flasks with the dilute bile salts, 0.33 per cent., and least in the most concentrated, *i.e.* 1 per cent. In order to determine the limits of this parallelism between the turbidity and the inhibiting action of the bile salts the following experiments were performed.

SERIES 12. Duration of experiments, 144 minutes. Temperature, 28° C. The quantity of ester solutions used in each case equalled 10 c.c. of a $\frac{N}{50}$ solution. The total volume was 20 c.c., and 1 c.c. of 10 per cent. clear liver extract was acting in each experiment. The numbers again indicate the percentage hydrolysis.

Ester.	Water.	Bile Salts. 6.67%	0.2%	0.02%	0.002%
Ethyl acetate	12.6	10.4	6.0	10.9	10.8
" propionate	52.7	51.7	6.2	44.4	51.5
" butyrate	65.4	70.5	9.4	73.5	72.2

The percentage inhibition caused by the bile salts in this series was as follows; the negative sign (—) before a number indicating acceleration:

Ester.	Bile Salts. 6.67%	0.2%	0.02%	0.002%
Ethyl acetate	17	52	13	14
" propionate	2	88	16	2
" butyrate	—8	86	—12	—10

This series brings out several additional points. The parallelism between the inhibition and turbidity was again borne out almost perfectly except in this, that the bile salts in 0.02 per cent. solution accelerated the action on the butyrate and still showed a faint turbidity which was less, however, than that observed with the other esters. In the 0.002 per cent. solution no turbidity developed. The statement previously made that the inhibiting action of the bile salts increases with increasing molecular weight of the acid has to be qualified. It only holds for the bile salts within a certain range of concentration (about 1 to 0.2 per cent. solutions). With greater concentration and

greater dilution the inhibiting action on the hydrolysis of all the ester is less, and decreases with increasing molecular weight of the acid, or even acceleration may be noted in the case of the butyrate.

The reason for the parallelism between the turbidity and the inhibition is obscure. As stated the bile salts are essential. The bile salts produced no turbidity with the enzyme nor with the ester alone, and no turbidity is developed in the mixture of all three if the solution is kept neutral. On acidifying a mixture of the extract and bile salts the turbidity quickly appears and it is undoubtedly due to the precipitation of some constituent of the extract by the bile acids. Maly and Emich[1] made a careful study of the precipitation of proteids by the bile acids and showed it to be an extremely delicate test for certain proteids. The simple production of a turbidity would not inhibit these processes since they occur readily in the turbid extract. Something essential to the process must be rendered functionless in the production of the turbidity. Further work along this line is contemplated.

THE EFFECT OF HEAT ON PANCREAS POWDERS.

The effect of heating the dry pancreas powders was studied in order to determine whether the activity toward ethyl butyrate and olive oil would suffer equally and disappear simultaneously. In 1892 Jacobson[2] employed a similar method to determine whether the power of almonds to decompose hydrogen peroxide is to be attributed to emulsin or to the presence of another ferment. He found that the power to decompose hydrogen peroxide is more readily lost on heating these preparations than is the emulsin, and concluded that the catalysis of hydrogen peroxide is due to a separate enzyme. Subsequent work has shown the correctness of this view.

0.5 gm. of pancreas powder was heated at 110° C. for $1\frac{1}{2}$ hours in an air bath. It was then removed, cooled, and ground up with 50 c.c. of

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[1] *Monatsh. f. Chem.*, iv, p. 89, 1883; vi, p. 95, 1885.
[2] *Zeitschr. f. physiol. Chem.*, xvi, p. 340, 1892.

water to a homogeneous suspension. A similar suspension was prepared from the same specimen of powder unheated.

(1) 5 c.c. *suspension of unheated powder.*
0.26 " ethyl butyrate.
0.1 " toluene.
(2) Duplicate of (1).
(3) Same as (1), but suspension of heated powder used.
(4) Duplicate of (3).
All acted for one hour at 40°.

(1) required	1.55	c.c.	$\frac{N}{20}$	NaOH
(2) "	1.45	"		"
Average of (1) and (2)	1.5	"	"	"
(3) required	0.75	"	"	"
(4) "	0.6	"	"	"
Average of (3) and (4)	0.67	"	"	"

Decrease in activity towards ethyl butyrate 55 per cent.

Action of the Above Suspensions on Olive Oil.—Each flask contained 10 c.c. of suspension, 0.3 gm. of olive oil and 0.2 c.c. of toluene. After acting at 40° C. for 18 hours 20 c.c. of neutralized alcohol were added and the titration with $\frac{N}{20}$ alcoholic KOH carried out, using phenolphthalein as the indicator.

(1) Suspension of unheated powder required	10.0	c.c.	KOH
(2) " " " " "	10.3	"	"
(3) " " " " "	10.3	"	"
Average	10.2	"	"

(1) Suspension of heated powder required	10.65	c.c.	KOH
(2) " " " " "	9.8	"	"
(3) " " " " "	10.15	"	"
Average	10.2	"	"

Decrease in activity towards olive oil 0.0.

Thus when heated at 110° C. for 1½ hours this particular pancreas powder retained only half of its activity on ethyl butyrate, while its action on olive oil remained undiminished. In a large number of experiments in which the pancreas powders were heated for varying periods of time at different temperatures the activity on the esters of the lower fatty acids was uniformly found to be more sensitive to heat in this dry state than the activity towards the fats. In one case in which the activity towards triacetin was also studied, the decrease in this activity was intermediate between the other two but was somewhat nearer that of the butyrate. Another striking fact brought out by these experiments

is the comparative stability of these enzymes toward heat in this dry powder form. The lipolytic enzyme has always been thought to be one of the most unstable of enzymes, but in the dry state it seems to be about as stable towards heat as other enzymes. After heating the powder at 125° C. for 35 minutes the activity towards ethyl butyrate was decreased 79 per cent. and towards olive oil, 62 per cent.

Taylor[1] has shown that the lipolytic activity of the dry powder obtained from the castor bean is also very stable toward dry heat. As mentioned above the liver powders were always found to be less active than the corresponding preparations from the pancreas. It was found that the ester-splitting function of the liver powders is more sensitive to dry heat than that of the pancreas powders.

DISCUSSION OF RESULTS.

Before considering the question of the identity of the enzymes concerned in the hydrolysis of various esters in the light of the results here presented, it is necessary to define the term enzyme, or at least for the writer to state what he understands that term to mean. That there are differences of opinion as to what is meant by the term is indicated by the recent controversies on the identity or non-identity of certain enzymes. Preparations from various living tissues have been shown to accelerate the hydrolysis of certain substances.[2] It has been generally supposed that the acceleration of a reaction caused by a given preparation is due to a substance contained in the mixture which is called an enzyme. As a matter of fact it never has been shown that only one substance contained in the preparation is responsible for the acceleration, and the generally prevailing idea is in part responsible for the confusion. It has been found that a solution possessing such an accelerating action usually retains its activity after prolonged dialysis. Hence the statement is made that the enzyme is not a dialyzable substance. It has been found that on boiling a solution possessing such an

[1] *Univ. of Cal. Pub., Path.*, i, p. 33, 1904.

[2] The discussion is here confined to the hydrolytic enzymes.

accelerating action the activity is lost. Hence the statement is made that the enzyme is destroyed by boiling. Now it is perfectly obvious that the word enzyme in the sense here used really signifies a certain property of the mixture, or the mixture which possesses that property and which can only be recognized by that property, without any reference whatever to the number of substances present which are essential to it. It is perfectly proper to say that on boiling saliva the property of rapidly hydrolysing starch is lost and this is all that the experiment shows. It must be borne in mind that in all the enzymic preparations hitherto obtained, we have in addition to the catalytic substance or mixture of substances, *i.e.* the enzyme, an unknown quantity and number of admixtures the effect of which must for the present remain unknown. This admixture i often of the greatest importance. Thus the work of Magnuss and the writer has shown that, in addition to the enzyme, the bile salts are essential in the hydrolysis of amyl salicylate by liver extract. Similarly an acid is essential in the action of pepsin on proteids. Chamoz and Doyon[1] found that pancreas extract does not hydrolyse amyl salicylate and therefore concluded that it does not contain the necessary enzyme, and that the enzyme here concerned is different from that which hydrolyses monobutyrin. Now it is quite possible that the pancreas does contain the enzyme but that in the absence of the bile salts it is unable to manifest itself, and in the light of recent results it would be rather hazardous to say that the enzyme which hydrolyses amyl salicylate is not the same as that concerned in the hydrolysis of monobutyrin. The physiologist is interested primarily in the processes which occur in a given organism or locality under the conditions which obtain there, and the existence of an enzyme in a tissue without the conditions necessary for its action is to the physiologist a matter of interest but is of secondary importance. He is interested in the combined effects of all the substances found there. In this sense, therefore. physiologists may look upon the bile salts as an essential part of the enzyme which hydrolyses amyl salicylate. Similarly in testing for the presence of pepsin in the tissues it is necessary

[1] *Loc. cit.*

to add hydrochloric acid or some other acid. The writer thinks that in these processes the bile salts and the acid which can be readily separated from the enzymic preparations should be called coferments.

All of the hydrolytic enzymes at present known are destroyed by heat, are non-dialysable and are catalytic in their action so far as this has been studied. It is to such a mixture of substances that the term enzyme should be restricted at present, and the term, coferment, can designate those substances which fail to have any of the above properties but which are essential to the action of the above group. The word enzyme is used in this paper only in the sense of this definition.[1]

From the above discussion the great difficulty of dealing with the question of the identity or non-identity of the enzymes concerned in the hydrolysis of the various esters, is perfectly apparent. We have first to consider whether the ester-splitting enzyme of the liver is identical with that of the pancreas, and then whether the hydrolysis of different esters by liver and pancreas is to be attributed to a single enzyme in each case. The enzymes of the liver and pancreas present certain resemblances and striking differences. The actions of both are remarkably sensitive to sodium fluoride. In ordinary concentrations this substance greatly inhibits the action of pancreas or liver extract on all of the esters studied. This is the most striking resemblance which this work has brought out. Furthermore all the esters here studied which are hydrolysed by the liver are also hydrolysed by the pancreas.

The most striking difference which has been made out between pancreas and liver extracts may be stated as follows. When the activity of the liver extract on the esters of the lower fatty acids is compared with that of the pancreas it is found that the liver is several times as active as the pancreas, but when the

[1] It is interesting to note in this connection Kühne's reason for introducing the word enzyme and what he denoted by that term. "Um Missverständnissen vorzubeugen und lästige Umschreibungen zu vermeiden schlägt Vortragender vor, die ungeformten oder nicht organisirten Fermente deren Wirkung ohne Anwesenheit von Organismen und ausserhalb derselben erfolgen kann, als Enzyme zu bezeichnen."—*Verhandl. d. naturh. med. Verein Heidelberg*, N. F., i, p. 190, 1877.

higher fats are used in making the comparison the activity of the liver is but a small fraction of that of the pancreas. It was shown that this difference cannot be due to the conditions prevailing in the liver because a mixture consisting of 90 per cent. liver extract and 10 per cent. pancreas extract is a very favorable medium for the action of the pancreas on olive oil. These observations seem to necessitate the view that the ester-splitting enzymes of the liver and pancreas are different. Various other differences were noted but these may be attributed to differences in the environment of the enzymes. Such for instance is the fact that it is very easy to obtain the enzyme of the liver in a clear solution, while this was not very successfully done in the case of the pancreas. Again liver extract is far more sensitive to the inhibiting action of bile salts and sodium fluoride than pancreas extract and finally the liver powders were rendered inactive at a lower temperature than the pancreas powders.

Having concluded that the enzymes of the liver and pancreas are different, we have now to consider whether each of these tissues contains a single enzyme which hydrolyses all of the esters or whether the hydrolysis of each ester is due to a separate enzyme. It is possible also that there may exist an enzyme which hydrolyses only one group of esters and that these tissues contain separate enzymes for the different groups of esters upon which they act. Here again certain facts speak for and others against each of these views. The following facts favor the view that the hydrolysis of each ester is due to a separate enzyme:

(1) A study was made of the activity of similar liver extracts on several esters and the activity in each case was calculated in terms of the activity on ethyl butyrate. It was found in this way that the relative hydrolysis of two esters may vary with similar extracts of livers of different individuals. Thus in one case an extract of a dog's liver hydrolysed one-third as much triacetin as ethyl butyrate, while a similar extract of the liver of another dog hydrolysed the two esters equally well. Similar variations were noted when pancreas extracts were used. The differences are far more striking with different species than with different individuals of the same species. Thus with dog liver extracts the hydrolysis of triacetin averaged 64 per cent. of that noted with ethyl butyrate, while with similar extracts of

beef liver the hydrolysis of triacetin averaged 203 per cent. of that of the butyrate.

The conclusion from these experiments then is that the activity of extracts of the same tissue obtained from different individuals and species may vary in their activity toward one ester more or less independently of the activity toward another ester.

(2) The effect of sodium fluoride and of bile salts on the activity of an extract varies with different esters. In these experiments the concentration of the fluoride and the bile salts plays a very important part. Thus the action of a pancreas extract on triacetin can be greatly inhibited by the fluoride, while the action on olive oil will be accelerated under the same conditions. Similarly the concentration of the bile salts required to accelerate the action of pancreatic juice on olive oil greatly inhibits its action on triacetin.

(3) On heating the dry pancreas powders at 110°–115° C. the activity toward ethyl butyrate suffers much more than its activity toward olive oil.

The following facts favor the view that the hydrolysis of the various esters by liver are due to a single enzyme and that the same holds true for the pancreas:

(1) The qualitative effects of sodium fluoride, the bile salts and lecithin on the hydrolysis of all of the esters are the same although the quantities required to show corresponding effects may differ widely for different esters.

(2) A study of the ethyl esters of the fatty acid series showed that under nearly all conditions a certain regularity is seen as we pass from the lower to the higher esters. Thus the inhibiting action of the fluoride decreases as the molecular weight of the ester increases. The inhibiting action of the bile salts in 0.2–1 per cent. solution increases with increasing molecular weight of the ester while in greater and in less concentrated solutions the inhibiting action was found to decrease with increasing molecular weight of the ester. Ethyl esters of acetic, propionic, and butyric acid were used in this part of the work and the above statements are made only in reference to these three esters.

When the activity of a liver extract was compared to the activity of a pancreas extract using a series of methyl and ethyl esters it was found that the value, $\frac{L}{P}$, decreases as the molecular weight of the acid from which the ester is derived, increases. Thus the value of $\frac{L}{P}$ determined for certain extracts with ethyl acetate was found to be 10.9 while with methyl palmitate it was found to be 0.28. With the intermediate esters the values found lay between these limits and were in the inverse order to the molecular weight of the corresponding acids.

This regularity seen in the homologous series is inexplicable on any other grounds than that one agency is concerned in the hydrolysis of the esters belonging to the series. If each of these processes were independent we should certainly find irregularities in such a series of experiments. It would be extremely interesting to compare an homologous series of ethyl esters with a corresponding series of glycerine esters. This might prove whether or not the hydrolysis of the esters of both series represent the same enzymic function. The writer believes that it would indicate the identity of these processes.

If we assume that one enzyme hydrolyses all of these esters, the question arises how are we to account for the fact that the activity of a given tissue from different animals on one ester can vary independently of its activity on another ester? The writer takes the view that such variations are due to variations in the admixture of substances in the extracts. All that can be said to be proved is that all of these processes show certain resemblances and certain points of difference.

SUMMARY.

1. The activity of the same tissue from different animals on one ester may vary independently of the activity on any other ester within certain limits.

2. The ratio of the activity of the liver to that of the pancreas ($\frac{L}{P}$) when a given ester is used varies to a certain extent with different individuals of the same species and to a greater extent with different species.

3. Using the same liver and pancreas extracts (pig) throughout, the value of $\frac{L}{P}$ was determined for a series of methyl and

ethyl esters of the fatty acids. It was found that the value of $\frac{L}{P}$ varies for different esters. Ethyl acetate gave the highest value (10.9) and there was a gradual decrease as the molecular weight of the corresponding acid increased, until with methyl palmitate the value found was 0.28. For olive oil the average value of $\frac{L}{P}$ was 0.14.

4. Pancreatic extract and pancreatic juice are more active on triacetin than on diacetin, and usually more active on diacetin than on ethyl acetate. Liver extract is much more active on the latter ester than on the acetins.

5. The action of mixtures of liver and pancreas extracts on ethyl butyrate is equal to that of the ingredients of the mixture when acting separately. The action of such mixtures on olive oil is far greater than that of the ingredients the acceleration in some cases being over 100 per cent. It is impossible to say whether the liver accelerates the pancreas or *vice versa* but the acceleration is not related to the high activity of the liver on lower esters because extracts of the testicle and spleen which have very little action on ethyl butyrate similarly accelerate the hydrolysis of olive oil when mixed with pancreas extract.

6. Bile salts in the proper concentration accelerate the action of pancreatic extract on ethyl butyrate, triacetin, and olive oil, but the accelerations noted were much less than was observed with pancreatic juice.

7. Bile salts in 0.2 to 1. per cent. solution greatly inhibit the action of clear liver extract on aqueous solutions of ethyl acetate, ethyl propionate and ethyl butyrate and the inhibition increases with increasing molecular weight of the ester. When employed in greater or in less concentrated solutions the inhibiting action of the bile salts decreases with increasing molecular weight of the ester. In all of the experiments the solutions became turbid and there was a remarkable parallelism between the extent of the turbidity and the inhibition noted.

8. Regarding the identity or non-identity of the enzymes concerned in these processes the tentative conclusions reached are as follows:

a. All of these processes exhibit certain similarities and differences.

b. The action of the liver and pancreas on all of the esters here studied is probably to be attributed to a single enzyme in each of these tissues.

c. The ester-splitting enzyme of the liver differs from that of the pancreas.

THE FATE OF RADIUM AFTER ITS INTRODUCTION INTO THE ANIMAL ORGANISM, WITH SOME REMARKS ON THE EXCRETION OF BARIUM.

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(Received for publication, October 31, 1906.)

In this paper are given the results of the third completed research with radium in a series of investigations now in progress.[1] The effects of intravenous injections of radium on blood pressure and respiration,[2] and also the influence of radium on metabolism, having been determined,[3] it seemed desirable to study the distribution and fate of radium in the animal body. The comparative ease with which infinitesimal amounts of radium can be traced—thanks to the delicacy of the physical methods for its detection—makes a study of its absorption and elimination very satisfactory.

ANIMALS AND METHODS.

Among the *animals* used for this investigation were those employed in the experiments of Burton-Opitz and Meyer and of Berg and Welker. Besides these a number of small dogs and cats were treated in various ways with radium preparations. In the use of the latter animals we avoided any disturbing factors, such as long continued narcosis and restricted diet, which might have influenced absorption and elimination in the former. The leading tissues and fluids of eighteen animals were examined. In some instances practically all the important parts of the same animal, and in others only the organs most likely to give positive results, were submitted to examination.

[1] Gies and collaborators, *Proc. of the Soc. for Exp. Biol. and Med.*, ii, p. 86, 1905; also, *Science*, xxi, p. 988, 1905; *Amer. Med.*, ix, p. 1029, 1905, and *Med. News*, xlii, p. 520, 1905.

[2] Burton-Opitz and Meyer, *Journ. of Exp. Med.*, viii, p. 245, 1906.

[3] Berg and Welker, this Journal, i, p. 371, 1906.

The *methods used for determining radioactivity*, although very delicate, do not permit of quantitative determinations of radium. The methods are mainly based upon the ionizing action of the rays on the surrounding gas. For masses of low activity, or small amounts of highly active material, two methods are commonly employed, one of them depending on the use of a gold leaf electroscope, the other of an ionization chamber in connection with a sensitive quadrant electrometer. The latter method is preferable. It is more rapid and will give an approximate indication of the relative radioactivity. That no accurate determination of the amount of radium present in a mass can be made by these methods is evident from the fact that the ionizing effects of radioactive substances are due to the various rays that are projected. The rays which take the most active part in this ionization are at the same time most readily absorbed in transit.[1] Unless the radioactive substance is spread out in a uniform and very thin layer, only a portion of the rays can participate in the ionization process. It is practically impossible to obtain such conditions satisfactorily for quantitative determinations. Consequently, although it is possible to determine quantitatively by electrical methods the radiations which leave the surface of a mass, the amount of substance which gives rise to these radiations cannot thus be ascertained.

The *gold leaf electroscope* was employed in these experiments mainly for the purpose of testing the radioactivity of the tissues and fluids before incinerating them, preparatory to making determinations with the electrometer. Before making the determinations the solid samples were hashed and dried, first on a water bath and then at 110° C. in an air bath, until they were freed from all apparent moisture.

The electroscope especially constructed and employed throughout the work consisted of a brass box 9 centimeters square and 12 centimeters high. An insulated gold-leaf carrier was fitted into the center of the cover. The gold leaf itself was insulated by a sulfur bead. Contact with the gold leaf was made possible by

[1] Rutherford, *Radioactivity*, 2d ed., p. 41, 1905.

means of a fine brass wire free to move in a hard rubber socket which was fitted into the cover. On opposite sides of the box and level with the gold leaf were two glass windows. A sliding drawer 85 millimeters long and 65 millimeters wide was situated at the bottom of the box. Tinned iron trays were made to fit this drawer. Each of the hashed or fluid samples was placed in one of these trays and dried in it in order to prevent so far as possible the drawer from retaining traces of radioactive material or becoming radioactive by induction. A new tray was used for each sample to be tested. The drawer and the box were washed, from time to time, with very dilute nitric acid to remove all traces of emanation as well as of the radiations which invariably collect on the sides of a vessel after repeated introductions of radioactive materials. This deposit materially increases the natural "leak" of the apparatus. The leak due to natural causes was very slight; it was nevertheless invariably taken into account in determining the rate and extent of discharge. As these determinations with the electroscope were meant to be of a preliminary character, no special means were employed to charge the gold leaf always to the same potential. Simple contact with an electrified rubber rod was found to answer all practical purposes. The angle to which the gold leaf was carried was always approximately the same. The rate of discharge was watched through a telescope fitted with cross-hairs and arc.

The degrees of radioactivity shown by the various organs and excreta were determined by means of the *quadrant electrometer*. For such a determination the weighed sample was dried at 100° C. and incinerated in a porcelain crucible over a Bunsen burner. A new crucible was used for each sample. The ash was used for the test.

Radium salts when heated lose their activity on account of the escape of emanation.[1] Soon after cooling, however, the radium residue exhibits renewed radioactivity. Fresh emanations are quickly generated, and, after a month, the radioactivity is usually equal to its original value. Consequently, the heating of the specimens merely delayed the determination of radio-

[1] Rutherford, *loc. cit.*, p. 371.

activity. Radioactivity was not measured till several months after incineration, by which time the ash had presumably regained its full activity. The measurements were repeated after several months but no variation from any previous determination was ever detected. This observation shows that no appreciable losses of radium from the tissues occurred in carrying out the method described.

The quadrant electrometer for making the measurements was very similar to that described by Rutherford.[1] It consisted of a metal box for an ionization chamber, having in it two metal plates 15 centimeters square (one 5 centimeters above the other), insulated from the walls of the box. The lower plate was connected with a source of potential of about 400 volts to obtain saturation current; the upper one was connected to one pair of quadrants of a quadrant electrometer. When the tray containing radioactive material is placed on the lower plate in such an apparatus the air between the plates is ionized and a current flows between the plates causing a steadily increasing deflection of the electrometer needle. The rate of movement of the needle over the scale is proportional to the ionization current. It was found necessary in this work to decrease the sensitiveness of the electrometer by connecting with it a condenser having a capacity of $\frac{1}{1000}$ microfarad.[2] All the measurements were made under these conditions.

The *radium preparations* used in this work and in the experiments already described[3] were of 240, 1000, or 10,000 activity. The radioactivity of these preparations was determined by the electrometer and found proportional to the numerical estimates just given.

Observations of the Radioactivity of Various Organs after Administration of Radium Bromid Preparations of 240, 1000, or 10,000 Activity.

The dogs made use of in the experiments of Burton-Opitz and

[1] Rutherford, *loc. cit.*, p. 90.

[2] A condenser is said to have an absolute unit-capacity when it is charged to a potential of one volt by a quantity of one coulomb. A farad is the practical standard of capacity and is equal to 10^{-9} absolute unit. A *microfarad* is a millionth part of a farad.

[3] Burton-Opitz and Meyer, and Berg and Welker, *loc. cit.*

Meyer were the animals used in this particular series of observations. A varying number of injections were made into the facial vein in repeated doses of 1.8 milligram of the radium bromid preparation contained in 10 c.c. of distilled water. The animals were kept under light ether narcosis. Usually not more than an hour elapsed between the last injection and the death of the animal. Death was induced by bloodletting. The blood was withdrawn from the femoral artery. After death the animal was opened along the median line. Ligatures were applied to the main blood vessels of the liver, heart, and other vascular parts, so that these organs could be removed without spilling residual radioactive blood upon any of the other parts.

Care was also taken to remove practically all extraneous blood from most of the tissues by rapidly washing them with water, but irrigation of the vascular tissues was not carried very far. In the main, however, the radioactivity of the parts as summarized in Table I was almost wholly that of radium actually present in the nonvascular portions of them.[1]

The entire organs were usually taken for the tests of radioactivity. Determinations made with the gold leaf electroscope are indicated in Table I by the letters A and I, which designate respectively that the organ was radioactive (A), or inactive (I). The numbers refer to the measurements made by the electrometer method and represent the rates of discharge as read on an arbitrary scale in terms of equal divisions per second. As the apparatus was always kept in the same condition, the figures give fairly accurate indications of relative radioactivity.

The weights of materials used in these determinations are not mentioned. From what has already been stated it is evident that the numbers expressing the rates of discharge due to the degree of ionization are proportional to the radiation projected from the mass of the active substance, that is, the total amount less the amount absorbed. None of the methods for determining radioactivity is adapted to an exact determination of the amount of radium present.[2]

[1] See remarks on pages 466 and 468.

[2] See page 462.

TABLE I.[1]

	1	2	3	4	5	6	7	8
Activity of the radium preparation employed	240	240	240	240	1000	10,000	10,000	10,000
Weight of dog (kg.)	3.4	5.0	3.7	4.3	4.0	5.5	5.2	7.0
Amount of the radium preparation injected (mg. per kg.)	5.3	7.2	12.1	8.4	0.4	3.2	3.8	3.6
Total amount of the radium preparation injected (mg.)	18.0	36.0	45.0	36.0	1.8	18.0	19.8	25.2
Blood	A	A	3.1	A	2.3	A	A	33.0
Brain	—	—	—	I	—	0.6	0.3	—
Kidneys	A	3.0	2.25	2.3	I	1.6	A	A
Liver	0.16	1.2	1.3	0.2	I	3.0	0.4	A
Lungs	A	2.0	2.0	0.6	I	—	A	A
Muscle, heart	—	—	—	I	—	A	—	—
Muscle, leg	—	I	—	I	—	I	—	—

The results of these experiments indicated that the blood was in all cases the most radioactive. In the short time during which the radium circulated, some was always retained in the kidneys, liver, and lungs. Special attention may be directed to the results obtained after the injection of the radium preparation of 1000 activity. The amount injected in that particular experiment was only very slight, as the animal succumbed immediately after the first injection. The duration of that experiment was only twenty minutes. This period of time was insufficient for the transportation of appreciable quantities of radioactive material to any of the organs and only the blood was found to manifest radioactivity.[2] Injection of preparations of high activity caused nearly every organ in the body to become more or less radioactive.

Of special interest is the fact that the injection of the radium preparation of 10,000 activity caused excessive salivation. Some of this saliva was collected and was found to be very slightly radioactive. The tongue of the animal also proved to be

[1] For further data see also page 467.

[2] This observation made it evident that the process of removing extraneous blood by washing with water, as employed in this work, was sufficient to eliminate all detectable radium in the blood of an organ.

radioactive. Besides the organs mentioned in Table I, it was found that the injection of this highly radioactive preparation gave positive radioactivity to the bile, eyes, organs of generation (both male and female), pancreas, spleen, stomach and stomach contents. Only in one instance could any urine be obtained and this proved to be very highly radioactive.

In order to study further the distribution of radium it was deemed desirable to allow the radium to act for a longer period than was possible in the experiments just described. With this end in view, it was decided to carry out a special series of experiments in which the animals could be kept for several days after the administration of radium. Relatively small animals were selected for this purpose in order to prevent excessive use of the radium preparations. The question as to the *lethal dose of radium bromid preparations* of low activity was also determined in this connection.

In this series of three experiments on small cats, varying amounts of radium preparations dissolved in 2.4 c.c. of distilled water were injected intravenously. The injection into the femoral vein was made slowly by means of a small hypodermic syringe. During the short operation very light ether anesthesia was maintained. The wound was washed with a very small quantity of a 1 per cent. solution of phenol and finally with sterile 0.8 per cent. saline solution. The wound in each case healed rapidly. Twenty-four hours after the last injection the animals were chloroformed and the samples for the determination of radioactivity were collected and treated as already described. Table II presents the data in this connection.

TABLE II.

Exp No	*Animal* Body Weight Kg.	Radio-*activity of* the Radium Bromid Preparation Used.	Amount Injected, in *Milligrams* Days					Results.
			1	2	3	4	Total.	
XIV.	1.0	240	3	3	6	12	24	Recovered
XV.	0 9	240	12	—	—	—	12	Dead in 22 hrs.
XVI.	1.3	1000	12	16	—	—	28	Recovered

All ordinary preparations of radium salts contain barium salts.[1] Preparations of low activity, as made by the Curie method, consist chiefly of barium salts. The influence of the admixed barium was evident in the experiment just described. The fatal dose of the preparation of radium bromid of low activity (240) was not very different from the figures given by Bary[2] for barium chlorid, namely, 15 milligrams per kilo of body weight. There seemed to be an indication of adaptation when the radium preparation was repeatedly administered in small doses. In the experiments of Burton-Opitz and Meyer,[3] it was also shown that with an increase of the amount of radium in the radium preparations, *i.e.*, with a radium preparation of higher activity, the toxicity of the associated barium was partly overcome. It would of course be desirable to study this question with radium of highest activity and purity. The scarcity of this product as well as its prohibitive cost make such a study, at present at least, entirely out of the question.

The death of the animal in which the dose proved fatal was accompanied by symptoms identical with those evinced by the dogs treated with a lethal dose of barium bromid. Death was seemingly brought on by an inhibition of respiratory activity. A decided irregularity of the heart-beat was noticeable in each case. This irregularity was similar to that described by Burton-Opitz and Meyer.[4] It soon returned to normal, however, in the animals that recovered. It was very pronounced shortly before death in the one fatal case.

The results of my determinations of the radioactivity of various parts of the three cats show that 24 hours after intravenous injection of radium bromid preparations of 240 activity, the blood no longer gave any indications of the presence of radium.[5] The feces and the urine (taken from the bladder after death), how-

1 Mme. Curie, "Untersuchungen über die Radioactiven Substanzen," *Die Wissenschaft*, i, p. 25, 1904.

2 Bary, "Beiträge zur Baryumwirkung," Inaug. Dissert., Dorpat, 1888, p. 19.

3 Burton-Opitz and Meyer, *loc. cit.*, p. 251.

4 Burton-Opitz and Meyer, *loc. cit.*, p. 247.

5 See page 466.

ever, were both decidedly radioactive. The lungs and kidneys from the cat in which the injections proved fatal were somewhat radioactive. The results of the examination of the other organs were negative.

The liver and kidneys of the cat in which the injection proved fatal and of the one treated with the preparation of 1000 activity, showed some radioactivity. The same organs of the cat which recovered from the injection of the preparation of 240 activity gave negative results.

Besides the above data on the general distribution of radium after its *intravenous* injection it is desirable to consider at this point the results of a study of the *fate of radium bromid when introduced into the body through other channels*. For this purpose three small well nourished dogs were treated with radium bromid preparation *per os*, subcutaneously or intraperitoneally. In no instance was the dose of radium large enough to prove fatal. The animal was chloroformed twenty-four hours after the last administration, and the organs were separated and treated as already described. The following notes of these experiments summarize the results:

XI. Dog, male; weight, 1.9 kg.; 28.5 mg. of the preparation of radium bromid of 240 activity (15 mg. per kilo of body weight) enclosed in gelatin capsules were administered *per os*. for three consecutive days.

XII. Dog, female; weight, 1.45 kg.; 21 mg. of the preparation of radium bromid of 240 activity were injected subcutaneously daily for three consecutive days. Each injection caused pain, which subsided in 5 to 6 minutes.

XIII. Dog, male; weight, 3.7 kg.; 30 mg. of the preparation of radium bromid of 240 activity were injected intraperitoneally daily for two consecutive days. No ill effects were noticeable.

The results of the determinations of radioactivity show that the ultimate fate of the radium was not greatly influenced by the channel of introduction. The results are in fact very similar to those already obtained with the cats. Whereas the tissues and organs were in general not radioactive the feces and urine were both decidedly so. The kidneys in all three instances showed only slight radioactivity, as did also the stomach of the animal to which the radium was given *per os*.

In the experiments thus far described the time between the final administration of radium and the collection of the samples for the determinations of radioactivity was from one hour to twenty-four hours; and the longest period during which the radium could distribute itself among the various organs was several days.

Before drawing any conclusions from the results of these experiments it may be well to pass on to the last series of determinations to be considered in this paper.

The Radioactivity of the Tissues and Organs of the Two Dogs Used by Berg and Welker in their Study of the Effects of the Administration of Radium on Metabolism.

In these experiments the radium preparations were administered *per os* or subcutaneously. The following facts which have a bearing upon the deductions to be drawn later are taken from the paper of Berg and Welker.[1]

METABOLISM EXPERIMENT III. A dog weighing 6.6 kg. was given *per os* during a period of 6 days a total of 1.0164 gm. of a radium bromid preparation of 240 activity; during 3 additional days 0.2497 gm. of a radium bromid preparation of 1,000 activity, and during 3 more days 0.1255 gm. of a preparation of radium bromid of 10,000 activity.

METABOLISM EXPERIMENT IV. A dog weighing 5.5 kg. was given three subcutaneous injections of radium bromid of 10,000 activity. A total of 0.0762 gm. was injected.

In both cases the dogs were chloroformed and parts taken for the radioactivity determinations *19 days after the last administration of the radium preparations*. The feces examined were those taken during a period of 14 days prior to the death of the animal, which occurred on the twentieth day after the last dosage.

The results of the determinations of radioactivity are summarized in Table III; column *A* gives the data for the parts after administration *per os*, whereas column *B* indicates radioactivity after subcutaneous injection.

[1] Berg and Welker, *loc. cit.*, pp. 400 and 404.

TABLE III.

	A	B
Blood	0.1	I
Brain	—	0.3
Feces	A	A
Intestines [1]	I	I
Intestinal contents	A	—
Kidneys	0.3	0.6
Liver	0.2	2.0
Lungs	A	1.0
Muscle, gastrocnemius	I	I
Muscle from place of injection	—	0.6
Pancreas	0.2	I
Spleen	I	I
Stomach	0.1	0.6
Urine	A	A

The urine examined was a fraction of the composite urine of the second period.[2] The feces were subject to daily examination after the last period of the metabolism experiment, and the statement in the table that they were found to be active applies to the feces generally after the treatment with radium.[3]

Table IV shows the results of my determinations of the radioactivity of the feces from both dogs beginning with the sixth day after the last administration of radium. In Column *A* are given the results obtained with the feces from the dog to which radium was administered *per os*, in Column *B* those of the dog treated subcutaneously.

TABLE IV.

No. of Days after the Last Administration of the Radium Preparation.	A		B	
	1 Wt. of Dry Feces. Gms.	2 Electrometer Divisions. Per. Sec.	1 Wt. of Dry Feces. Gms.	2 Electrometer Divisions. Per. Sec.
6	0.7	3.6	18.6	10.9
7	25.0	20.0	0.0	—
8	0.2	2.5	8.4	1.2
9	0.6	2.0	7.6	1.3
10	14.9	8.0	4.6	0.3
11	21.8	6.5	10.2	0.6
12	19.1	5.0	5.6	0.5
13	9.5	1.1	7.6	0.8
14	4.5	0.5	7.9	0.6
15	3.8	0.4	5.9	1.2
16	17.0	2.5	7.4	0.4
17	10.4	0.2	9.1	0.3
18	0.0	—	2.0	0.2
19	2.5	I	5.2	0.3

[1] See foot-note page 473.

[2] See Berg and Welker, *loc. cit.*, pp. 400 and 473.

[3] See concluding paragraph of this paper.

DISCUSSION OF RESULTS OBTAINED WITH RADIUM.

The results of this investigation show that radium even when introduced in small amounts is absorbed and carried to practically all parts of the body. Its subsequent presence in the various parts is influenced by the channel of introduction, the amount given, and the lapse of time after its administration and before the death of the animal. Control determinations carried out on dogs treated with barium chlorid gave no indications of radioactivity in any fluid or tissue.

When introduced in very small amounts directly into the circulation, radium passes but slowly to other tissues. Only the blood will give a positive test for radium if the examination is carried out a few minutes after the injection. The degree of radioactivity of the preparation injected influences this result, of course. After injections of very highly active material all organs are more apt to receive radium immediately than if a less active preparation had been used. The blood gradually loses its radioactivity; the organs in turn become more and more radioactive and then release their radium continuously. The liver, lungs, and kidneys appear to be among the first organs to show the presence of radium after its intravenous injection. Radium seems to pass promptly into the urine and into the intestinal canal.

The ultimate fate of radium introduced subcutaneously, intraperitoneally, or *per os* is not materially different from that of radium introduced intravenously. The extent of distribution invariably depends on the strength of the preparation and the time it has to act. Of special interest in this connection are the results of the determinations of radium in the organs of the animals used in the metabolism experiments of Berg and Welker.[1] Radium of 10,000 activity was used in both of the experiments, and the animals were seemingly kept long enough (twenty days after the last administration) to allow the radium either to reach all parts of the body or to be entirely eliminated. It was found, as in the previous instances, that the blood contained but relatively slight amounts of radium, the other organs in

[1] See page 470.

general being more radioactive.[1] The stomach of the animal to which radium had been given *per os* was less radioactive than the one treated subcutaneously. This would suggest that radium, like arsenic, antimony, manganese, bismuth, atropin, quinin, morphin, and other substances is eliminated into the stomach. Recently Lewin[2] found barium in the stomach wall after subcutaneous injections of barium chlorid.

It is not the intention of the writer to enter upon a detailed discussion of the elimination of radium. The results of the experiments here reported are hardly sufficient to warrant final conclusions[3] in this connection. Nevertheless a few facts may be noted.

Radium appears to be a member of the group of alkali earth metals. It is interesting to find that the facts regarding its elimination accord in a general way with those pertaining to calcium, magnesium and the remaining members of the alkali earth group. I have been particularly interested in comparing my data on the elimination of radium with the results obtained by other observers on the excretion of barium, especially because all my preparations of radium bromid contained barium bromid,[4] and also because previous observations on the elimination of barium have not been entirely in accord.

REMARKS ON THE EXCRETION OF BARIUM.

In their study of the metabolic influences of barium bromid

[1] See Table IV.

[2] Lewin, *Deutsche med. Wochenschr.*, 1906, p. 173.

[3] A more detailed study of this phase of the problem is now being carried on in this laboratory by Dr. W. Salant and the writer, the results of which will shortly be published.

[4] The radium preparations were made by the Curie method. "Radium is extracted from pitchblende by the process used to separate barium, to which radium is very closely allied in chemical properties. After the removal of other substances, the radium remains behind mixed with barium. It can, however, be partially separated from the latter by the difference in solubility of the chloride in water, alcohol, or hydrochloric acid. The chlorid of radium is less soluble than that of barium, and can be separated from it by the method of fractional crystallization. After a large number of precipitations, the radium can be freed almost completely from the barium." Rutherford, *Radioactivity*, 2d ed., p. 13, 1905.

and radium bromid Berg and Welker observed incidentally that *barium was not present in appreciable quantity at any time in the feces* of a dog that received large doses of barium bromid under the skin daily for several days—as much as 40 milligrams per kilo at one time. They also noted the fact that in another dog, which had received large amounts of barium bromid daily *per os*—as much as 175 milligrams per kilo—*barium was absent from the urine.* These facts were regarded by them as indications that very little barium was absorbed in the experiments in which those particular dogs were used. They drew no other general conclusions from *their own* results on the elimination of barium,[1] but that the reader might be reminded of other data they quoted contrary observations by previous investigators. From the results obtained by earlier workers in this connection they considered that barium, like calcium and magnesium, may be eliminated into both the feces and urine, in *some* animal, at least, after sufficient dosage, whatever its channel of introduction. The data of previous observers made any other conclusion impossible.

Shortly after the appearance of the paper by Berg and Welker, Mendel and Sicher[2] published the results of a few experiments on two dogs from which they drew even broader conclusions as to the excretion of barium in the urine after subcutaneous injection of the chlorid. Mendel and Sicher made no allusion to the observations of Berg and Welker in this connection.[3] With regard to Bary's important work they merely remarked that he "is said to have detected barium in the urine after subcutaneous administration of its salts." Their authority for this remark, as they indicated in a footnote, was Bary's dissertation, which they stated, however, was not accessible to them.

Berg and Welker gave on page 397 of their paper the following facts from a copy of Bary's dissertation, which was procured

[1] The results of a single observation in each case of course justified nothing more.

[2] Mendel and Sicher, *Amer. Journ. of Physiol.*, xvi, p. 147, 1906.

[3] They referred to Berg and Welker's related conclusions on radium however.

for the use of all the workers in this laboratory, at the time the radium investigations were inaugurated:

"A female dog which weighed 21.5 kilos was given a subcutaneous injection of 4 c.c. of 5 per cent. barium chlorid solution (10 mg. barium chlorid, equivalent to 16 mg. barium bromid, per kilo). *Five hours* after the injection, the dog was catheterized, and 60 c.c. of clear urine were obtained. Barium was present in the urine, but the author does not mention the quantity found. 'Bei der Veraschung des Urins, war eine deutliche Menge von Baryum vorhanden, welche spectroskopisch sicher gestellt war.' This is the only experiment (out of twenty similar ones) in which barium was found in the urine."

I have compared the foregoing quotation with the original and find that the facts given agree with it entirely.[1]

Bary's work justifies the conclusion that after subcutaneous injection of barium chlorid, barium appeared in the urine of one dog, and that it *may* or may not be eliminated under such circumstances in the urine of others. It seems to me that Mendel and Sicher were not warranted in drawing any broader conclusions in this connection from merely a single positive result with one dog, accompanied, as that result was, by a negative observation or its equivalent in a similar experiment on another.

The two experiments performed by Mendel and Sicher in this connection may be summarized as follows:

A. Bitch; weight, 11 kilos. 98 mg. barium (in the form of $BaCl_2.2H_2O$) injected subcutaneously. "Uncontaminated urine (275 c.c.) collected *after 18 hours* gave a strong test for barium.[2] *Diarrheal stools* gave a fair test. Urine (350 c.c.) collected on the *second* day gave no test for barium."

B. Dog; weight, 12½ kilos. 134 mg. barium (in the form of $BaCl_2.2H_2O$) injected subcutaneously. "In 100 c.c. of urine collected *on the*

---

[1] The last sentence of the above quotation might be taken by some to mean, however, that after many attempts to find barium in the *urine* of the animals, Bary succeeded only once. It is true that it was found in the *urine* of only one animal, but the description of his experiments does not make it evident that he examined the *urine* of more than that one animal for it.

[2] Mendel and Sicher stated that a detectable excretion of barium through this channel (kidneys) ceases within a few hours. The data on which this conclusion depends do not appear in their paper.

following morning no barium could be detected. A later portion *presumably containing some barium was lost* (!). Urine collected on the 4-6 days contained no detectable traces of the element.[1] There was, notably, *an absence of diarrhea* in the first days of this experiment and with the concentrated diet of meat no feces were obtained until the sixth day. They contained 7.8 mgs. of barium. On the eleventh day feces containing 7.7 mgs. of barium were again obtained.[2] Feces collected at varying intervals after this also gave distinct evidence of barium."

No other urinary data, except for *mixed urine and feces*,[3] were offered by Mendel and Sicher upon which to base their general conclusions on the elimination of barium in the urine. I fail to see any confirmation of the essential urinary results of their first experiment by anything that was recorded for their second. The presumption that barium was present in the particular portion of the urine which was lost in spite of the fact that barium could not be detected in any other sample of the same dog's urine, is no addition whatever to experimental evidence on the subject. The positive result of their first experiment confirmed Bary's observation.

The italics in the above summaries are mine. It will be noticed that the animal that excreted barium in the urine also eliminated diarrheal stools. On the other hand the second animal, after receiving a much larger dose of barium chlorid, eliminated fecal matter that was characterized by a "notable" absence of diarrhea and excreted urine which, so far as its examination went, was *free from barium*. The average amount of barium excreted *per diem* in the feces of the second animal was apparently only about 1 per cent of that administered.

Berg and Welker observed considerable variation in the responsiveness of their animals to the barium treatment, just as Mendel and Sicher and others did. Berg and Welker were unable to detect barium in the feces of a dog which had received daily for some time comparatively large amounts of barium bromid subcutaneously. There was a notable absence of diarrhea from the stools of that particular dog.

1 Nothing was said about the urine portions of the first three days except the remarks quoted.

2 Only 1.4 mg. per day for 11 days.

3 The barium in such a mixture could not, of course, be accredited to the urine.

We believe in this laboratory that there is considerable variation in the susceptibility of animals to the influence of moderate amounts of barium salts, and similar uncertainty in some respects regarding the direction, rate, and amount of excretion of barium after such dosage. It seems probable that if diarrhea ensues after subcutaneous introduction of barium salts, sufficient barium has been absorbed from the injected mass not only to pass into the intestine in relatively large amounts but also to yield a detectable proportion to the urine. When diarrhea does not result possibly the quantity of absorbed barium, while still sufficient to yield appreciable quantities to the intestine, is insufficient nevertheless to yield detectable quantities to the urine. I am carrying out a series of experiments to ascertain the facts in this and related connections.

In my work with radium, I have been favored by the fact that the presence of quantities far too slight for detection by any chemical means could be proved easily with the aid of an electrometer. It is quite as possible that more refined methods for the detection of barium would invariably reveal its presence in the urine for a given period after its introduction *per os* or by any other channel. Both the urine and feces have been found to be radioactive, whatever the channel of introduction. In each of the metabolism experiments by Berg and Welker, even 19 days after the last administration, the feces were still radioactive.

Whether the kidney or the intestine is the principle route of radium elimination cannot be stated from the data presented.[1] That the intestinal contents and feces contained radium at the same time that the intestinal walls gave no evidence of its presence after subcutaneous injection seems apparently contradictory. However, radium may actually have been present in the intestinal membrane, but the amount contained in the latter at any moment may have been beyond the limits of delicacy of the methods for its detection. Again, the determination of comparative radioactivity (Table III) may have been made after the complete elimination of radium by the intestine. On the other hand, it is possible that radium fails to pass directly through

[1] See foot-note p. 473.

the intestinal wall. The elimination of radium may be restricted to certain parts of the gut, a matter of investigation that was not included in these tests. The presence of radium in the gastro-intestinal tract under the conditions of such experiments as these may depend mainly, perhaps entirely, on its secretion in the digestive juices. Thus, I have found that the contents of the gall bladder removed at autopsy in one of the foregoing experiments were radioactive. Likewise the contents of the stomach have been found to be radioactive after *intravenous* injection of radium. Radium may also be eliminated in the saliva and thus find its way to the feces. The saliva in some instances was found to be highly radioactive. Further facts in these connections will be communicated after the conclusion of the experiments now in progress.

SUMMARY.

By whatever channel radium is introduced it seems to find its way into practically all the tissues and fluids of the body.

It is eliminated to a certain extent in the urine and largely with the feces, in which it leaves the body continuously in small amounts. Its excretion may therefore be continued over a long period. No observations were made on its removal through the skin or lungs.

The range of distribution of radium in the body is influenced somewhat by the channel of its introduction as well as by the radioactivity (purity) of its compound, and the time that elapses after its administration before the death of the animal.

So far as its distribution and elimination are concerned radium is like the alkali earth metals. Biological data conform with chemical facts in showing this resemblance. The results with radium suggest that if chemical methods for their identification were delicate enough, barium, calcium, and their metallic congeners could be detected in all parts of the organism after their administration and would be found under such circumstances in practically all the excreta.

Certain results that confirm previous observations by Burton Opitz and Meyer and by Berg and Welker were considered.

Recent observations on the excretion of barium have been discussed.

The foregoing research was suggested by Dr. William J. Gies. I wish to express my obligations for his many valuable suggestions and helpful criticisms. Thanks are also due to Mr. Hugo Lieber who generously supplied the radium preparations needed in the work. The physical determinations with the electrometer were carried out in the Department of Physics of Columbia University. I am greatly indebted to Dr. G. B. Pegram for his valuable advice on matters pertaining to the measurements of radioactivity.

ON THE QUANTITATIVE ESTIMATION OF TRYPTOPHAN IN PROTEIN CLEAVAGE PRODUCTS.

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(Received for publication, November 29, 1906.)

The method of Hopkins and Cole[1] made possible the isolation of tryptophan from the other protein cleavage products. The methods of preparation of the pure substance always lead to appreciable loss and the yield of the pure substance does not furnish exact information regarding the real quantity of this constituent in the protein molecule. It seemed, therefore, desirable to devise a method by which tryptophan could be estimated quantitatively. A method which naturally suggested itself is the colorimeter, which, as is the case with all similar methods, is very imperfect. We preferred to resort to a method based on the following observation. When bromine water is added to a tryptophan solution a purple coloration develops. The intensity of the color increases with the continued addition of bromine water until a maximum is reached. At this phase the solution becomes very sensitive to further addition of the reagent. As little as one additional drop of bromine water causes the disappearance of the purple color. The nature of the chemical changes, which bring about the described reactions, will be studied in the future.

In applying this property of bromine water in the quantitative estimation of tryptophan, one must bear in mind that there are several other products of protein cleavage which combine with bromine, and some of them form colored substances, the presence of which renders it difficult to distinguish the end point of the tryptophan reaction. In order to obviate this difficulty it was found necessary to titrate, not the solution containing all the products of protein cleavage, but only the fraction precipitated by the reagent of Hopkins and Cole.

[1] *Journ. of Physiol.*, xxvii, p. 418, 1901.

The process of tryptophan estimation in detail is then as follows: The solution of the products of protein digestion or hydrolysis is made to contain 5 per cent. of sulphuric acid and is then treated with the mercuric sulphate reagent[1] of Hopkins and Cole, which yields a precipitate. The reagent is added gradually, the supernatant liquid being tested with bromine water from time to time. It is preferable to end the addition of the bromine just at the point at which the supernatant liquid ceases to form the purple coloration. The mixture is then allowed to stand for twenty-four hours. The precipitate is filtered off, suspended in water containing not more than 1 to 2 per cent. of sulphuric acid, and decomposed by sulphuretted hydrogen. The filtrate from mercuric sulphide is heated on the water bath until all hydrogen sulphide is removed, brought to a definite volume, and is then ready for titration. Fifteen cubic centimeters of the solution are taken in a test tube for analysis. To this are added 2 cubic centimeters of amyl alcohol which serve for the extraction of the coloring matter. The bromine water is added gradually and the tube is shaken vigorously. The addition of bromine water is discontinued as soon as the purple color of the amyl alcoholic layer disappears. Duplicates of the same solution on titration consumed quantities of bromine water differing by from 0.05 to 0.10 cubic centimeter of the reagent.

The concentration of the tryptophan, and the degree of acidity of the solution employed for analysis remain without influence on the end reaction. This can be seen from the following experiments.

I. INFLUENCE OF CONCENTRATION.

Tryptophan solution	10 c.c.	5 c.c.
Water	5 "	10 "
Bromine water	1.85 "	0.9 "

(2 c.c. of amyl alcohol and 6 drops of a 15 per cent. solution of sulphuric acid were added to each tube.)

II. INFLUENCE OF ACIDITY.

Tryptophan solution	10 c.c.	10 c.c.
Water	5 "	5 "
Amyl alcohol	2 "	2 "
Sulphuric acid, 15 per cent.	6 drops	—
Sulphuric acid conc.	—	6 drops
Bromine water	1.85 c.c.	1.75 c.c.

1 Ten per cent. of mercuric sulphate, dissolved in a 5 per cent. solution of sulphuric acid.

Since, however, the precipitate obtained by Hopkins' reagent contains cystin and tyrosin in addition to tryptophan, it was necessary to inquire into the influence of these substances on the titration with bromine. It was found that each of the two substances combines with bromine.

Tyrosin solution	5 c.c.	—
Water	10 "	15 c.c.
Amyl alcohol	2 "	15 "
Sulphuric acid, 15 per cent.	6 drops	6 drops
Bromine water	0.5 c.c.	0.15 c.c.

Titrated until the amyl alcohol layer acquired a pale yellow coloration. Thus 5 cubic centimeters of the tyrosin solution consumed 0.35 cubic centimeter of the bromine water.

The bromine water consumed by a solution containing both tryptophan and tyrosin is equal to the sum of the quantities required for titration of each of the substances separately. This is seen from the following experiment.

Tryptophan solution	10 c.c.	10 c.c.	10 c.c.
Tyrosin	—	2.5 "	5 "
Water	5 "	2.5 "	—
Amyl Alcohol	2 "	2 "	2 "
Sulphuric acid, 15 per cent.	6 drops	6 drops	6 drops
Bromine water	1.85 c.c.	2.00 c.c.	2.2 c.c.

Deducting the bromine water required for saturation of the tyrosin one finds that in every one of the three experiments the tryptophan required 1.8 cubic centimeters for its saturation.

It should be remarked, however, that if precautions are taken the quantity of tyrosin present in the tryptophan fraction can be reduced to a mere trace. This is accomplished first by avoiding the addition of an excess of the mercuric sulphate solution. As already mentioned, the addition of the reagent is discontinued as soon as the remaining solution ceases to form the typical coloration with bromine water. The precipitate is to be washed with a 5 per cent. solution of sulphuric acid until the wash water contains no tyrosin. One can calculate the quantity of tryptophan in the presence of tyrosin by making a nitrogen estimation of the solution.

Of greater importance is the presence of cystin as seen from the following experiment.

Tryptophan solution	5 c.c.	5 c.c.	—
Cystin (solution of sodium salt)	—	5 "	5 c.c.
Water	10 "	5 "	10 "
Amyl alcohol	2 "	2 "	2 "
Sulphuric acid, 15 per cent.	6 drops	6 drops	6 drops
Bromine water	0.95 c.c.	1.75 c.c.	0.8 c.c.

(The tryptophan solution contained 0.0926 gm. in 100 c.c. of water; of tyrosin 0.069 gm. in 50 c.c.; of cystin also 0.0708 gm. in 100 c.c.)

Since the quantity of cystin can be easily estimated by a sulphur determination there is no difficulty in calculating the quantity of tryptophan present in the solution. As already mentioned the quantity of tyrosin in the tryptophan fraction can be reduced to a mere trace, and since the solution binds but little bromine by its presence, can be disregarded. In estimating the tryptophan one should proceed in the following manner: (1) Titrate the solution of cystin and tryptophan; (2) In an aliquot part of the solution make a sulphur estimation and calculate the quantity of bromine required to saturate it; (3) Deduct the last figure from that obtained on titration of the solution containing both substances and the resulting figure represents the number of cubic centimeters of bromine water required to saturate the tryptophan. It is an advantage to standardize the bromine water with solutions of tryptophan and cystin before each analysis.

A study of the conditions giving the best yield of tryptophan on protein cleavage, and of the quantity of the substance present in various proteids is now in progress.

A NOTE ON THE PRESENCE OF LACTIC ACID IN THE URINE OF PERNICIOUS VOMITING OF PREGNANCY.

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(Received for publication, Dec. 7, 1906.)

That the study of the distribution of the various forms of nitrogen in the urine in pathological conditions may be of practical importance has been repeatedly demonstrated. In diabetes, etc., the changes in the distribution of urinary nitrogen are too well-known for repetition here. More recently, however, marked variations in the distribution of the nitrogen of the urine have been reported in abnormal conditions attendant upon pregnancy. Thus Zweifel [1] has demonstrated an increase of ammonia at the expense of the urea in eclampsia; and Williams[2] has found like conditions existing in certain types of pernicious vomiting of pregnancy.

The object of this paper is to present a report of a case of pernicious vomiting of pregnancy. The patient[3] had had two normal pregnancies, and when she first came under my attention was in her fifth pregnancy and suffering from a pronounced pernicious vomiting. The two previous pregnancies had been of the same type and vomiting ceased only after the uterus had been emptied. At the time the following experiments were undertaken she was in the second month of pregnancy, in the hospital, and being fed by rectal enemata only.

In the following table is given the determination of total nitrogen, urea nitrogen, and ammonia nitrogen for twenty-four

[1] Zweifel, *Arch. f. Gynaekol.*, lxxii, p. 1, 1904.

[2] Williams, *Johns Hopkins Hospital Bulletin*, xvii, p. 71, 1906; *Amer. Journ. of the Med. Sci.*, cxxxii, p. 343, 1906.

[3] I am greatly indebted to Dr. R. F. Rand for his courtesy in placing this patient at my disposal.

hour periods. The methods employed were those commonly in use in this laboratory.[1]

COMPOSITION OF THE URINE.

Date	Volume	Specific Gravity	Reaction to Litmus	Total N.	Urea N.	Ammonia N.	Urea N.	Ammonia N.
Oct.	c.c.			gm.	gm.	gm.	per cent.	per cent.
11	880	1.012	Acid	4.86	2.63	1.59	54.1	32.7
12	990	1.009	"	4.04	2.32	1.12	57.4	27.7
13	660	1.014	"	2.85	1.45	0.69	50.8	24.2
16*	1090	1.004	"	7.18	5.31	0.84	73.9	11.7
21	605	1.014	"	4.97	4.12	0.16	82.9	3.2

* Uterus was emptied on the evening of October 14.

The abnormally high percentage of ammonia at the expense of urea is in entire accord with some of the results obtained by Williams under similar conditions. According to the well-known theory of acidosis the high percentage of ammonia may be considered as indicative of an increased production of organic acids in the body. In diabetes β-oxybutyric acid is formed and ammonia may be utilized to neutralize it, and in eclampsia[2] lactic acid is produced and is probably excreted as the ammonium salt. So far as I am aware, no attempt has been made to determine what acid (or acids) may be formed in the organism in pernicious vomiting of pregnancy which is responsible for the withdrawal of ammonia. Such an attempt is recorded below.

Of the urine of Oct. 11, in which the ammonia content rose to 32 per cent., 500 c.c. were taken and the presence of lactic acid tested for according to the well-known method.[3] From the final aqueous fluid there separated on standing 0.68 gram of a zinc salt, having the crystalline form of zinc paralactate, and showing lævo-rotation. This salt was identified by a determination of the water of crystallization and the content of zinc.

Calculated for $Zn(C_3H_5O_3)_2+2H_2O$.		Found.
H_2O	12.9 per cent.	12.7 per cent
Zinc	26.7 " "	26.4 " "

[1] Underhill and Closson, this Journal, ii, p. 117, 1906.

[2] Zweifel, *Arch. f. Gynaekol.*, lxxvi, p. 537, 1905.

[3] Hoppe-Seyler-Thierfelder, *Handbuch der chemische Analyse*, 7th ed., p. 449.

From the urine of Oct. 21 not a trace of lactic acid could be obtained.

The quantity of the acid obtained on Oct. 11 (over a gram of the zinc salt for the 24 hours' urine) is relatively large, and whether its production is an etiological factor of the pathological condition noted or simply the result of an abnormal metabolism can be decided only by future investigation.

STUDIES OF THE EFFECTS OF IONS ON CATALYSIS, WITH PARTICULAR REFERENCE TO PEPTOLYSIS AND TRYPTOLYSIS.

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(Received for publication, November 3, 1906.)

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INTRODUCTION.

Five years ago the senior author of this paper began a study of catalysis under various ionic conditions, and, in the course of

two years, by occasional effort, finished about twenty-five experiments. Until lately, however, more urgent work interfered with the progress of the contemplated series of researches.

The main conclusions of two important sections relating to the work on *peptic* proteolysis were published nearly four years ago. The first results obtained in this connection were communicated (December, 1902) in a preliminary report on "the influence of the hydrogen ion in peptic proteolysis." [1] About five months later (May, 1903), the results of a second series of experiments, on "peptic proteolysis in acid solutions of equal conductivity," [2] were reported in a similar communication.

The experiments referred to were carried out by the senior author and were conducted with considerable attention to chemical and physical details connected with the digestive products. One of the first difficulties encountered in the effort to make the work as accurate as possible was the quantitative estimation of acidalbumin (neutralization precipitate). On account of this obstacle it was soon found desirable to study the quantitative determination of acidalbumin as a special problem. Accordingly, before proceeding with the main investigation, Dr. P. B. Hawk, then an assistant in this laboratory, was invited to co-operate in conducting the research which was described in the paper "on the quantitative determination of acidalbumin in digestive mixtures." [3]

In the paper describing the results of that investigation the following remarks were made (p. 460):

"In many of the experiments which have been carried out to determine quantitatively the proteolytic power of pepsin under various conditions, the chief deductions have been drawn directly from the amounts of undigested or residual matter rather than from the proportions of the digestive products themselves. In a majority of these cases the figures

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[1] Gies, *Proc. of the Amer. Physiol. Soc.*, 1902; *Amer. Journ. of Physiol.*, viii, p. xxxiv, 1903. Also Gies and collaborators, *Biochemical Researches*, i, p. 61, 1903.

[2] Gies, *Proc. of the Amer. Physiol. Soc.*, 1903; *Amer. Journ. of Physiol.*, ix, p. xvii, 1903. Also Gies and collaborators, *Biochemical Researches*, i, p. 62, 1903.

[3] Hawk and Gies, *Amer. Journ. of Physiol.*, vii, p. 460, 1902. Also Gies and collaborators, *Biochemical Researches*, i, Reprint No. 34, 1903.

for undigested matter have doubtless suggested approximately correct conclusions in this regard, but it seems probable that, in some instances at least, quantitative studies of the albuminates, proteoses, and peptones formed would have furnished more accurate and acceptable data.

"The writer has recently been engaged in a study of the action of pepsin under varying degrees of acidity with a number of acids, and in the presence of different ions, the results of which will be reported later. In experiments of such character the increasing or decreasing amounts of acid associated with the pepsin, to say nothing of its quality, variously affect the proteid indicator, irrespective of the influence on the latter of the enzyme. Different proportions of acidalbumin would be formed, also, with variations in the chemical character and physical condition of the proteid used to test relative zymolysis. If correct comparative deductions are to be drawn from the results of such experiments, it would seem that determining the amounts of albuminate present in each case would be almost if not quite as important as ascertaining the quantity of undissolved or undigested substance. It is conceivable that in comparative cases, where, for example, the undigested matter might be decreased, the proportion of acidalbumin formed by the mere solvent action of the acid might be correspondingly larger. To assume from the fact of diminished quantity of original proteid, in such an instance, that zymolysis had been greater in the one case than in the other obviously would be unwarranted."

All the peptic experiments reported in the present paper were carried out in the light of the information gained from the abovementioned experiments on the quantitative estimation of acidalbumin.[1] This information was helpful in the tryptic experiments also.

A few months ago resumption of the work and more detailed study of the several questions involved in it were made possible by the co-operation of the junior author. This paper gives the essentials of all the work done in this laboratory since

[1] In anticipation it may be said that in most of the experiments (with fibrin) described in this paper, our conclusions would have been practically the same with regard to rate and extent of zymolysis if we had had only data for residues (undigested substance). When proteins like "myosin" are used in such experiments, however, determinations of the amounts of neutralization precipitate would seem to be particularly desirable. The value of data for neutralization precipitate in such work will be further investigated with many other protein products in a research already inaugurated.

the winter of 1901–02 on peptic and also on tryptic[1] proteolysis.

Experimental.

A—Solutions, Enzyme Preparations, and Protein Indicators.

The essential products employed in each digestion experiment were (1) the aqueous solution containing $H^{\cdot}$ or OH' ions, (2) the material containing pepsin or trypsin, and (3) the indicator of proteolysis (protein product).

Acids and Bases.—All the solutions used in these experiments were prepared with the greatest concern for quantitative accuracy.

Acids.—Ordinary mineral and organic acids, most of which commonly occur in food as free acids or as salts, were selected. Their names and formulas are summarized below:

	Inorganic:	Organic:
Monobasic	Hydrochloric, HCl Hydriodic, HI Chloric, $HClO_3$ Nitric, HNO_3	Lactic, CH_3-CHOH-COOH Acetic, CH_3COOH
Dibasic	Sulfuric, H_2SO_4	Oxalic, COOH | COOH Tartaric, CH(OH)-COOH | CH(OH)-COOH
Tribasic	Phosphoric, H_3PO_4 Arsenic, H_3AsO_4	Citric, CH_2-COOH | C(OH)COOH | CH_2-COOH

We used the best acid preparations obtainable in the market. They appeared to be practically free from impurity except water. Equinormal (isohydric) stock solutions were very carefully prepared with the aid of $\frac{N}{10}$ sodium hydroxid solution. Titration was conducted with congo red or phenolphthalein, and numerous checks were made until the

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[1] Berg (communicated by Gies), Proc. of the Sect. of Biol. Chem. of the Amer. Chem. Soc. in affiliation with Section C (chemistry) of the Amer. Assoc. for the Adv. of Sci., December, 1905. *Science*, xxiii, p. 335, 1906. *Proceedings* of the Association, p. 331, 1906. Also Berg and Gies, *Proc. of the Soc. for Exper. Biol. and Med.*, iv, p. 17, 1906–07.

solutions were rendered assuredly equivalent,[1] directly or after simple dilution. The remaining types of acid solutions were prepared, as a rule, from the equinormal (isohydric) solutions[2] by proper dilution. Most of the *equidissociated* (*isohydrionic*) solutions, however, were very carefully prepared for us independently by Dr. C. W. Kanolt, in the laboratory of physical chemistry. There were two groups of such acids, representing two degrees of hydrion (H˙) concentration, *i.e.*, each was equal in hydrion concentration to that of a 0.09 per cent. or a 0.19 per cent. aqueous solution of hydrochloric acid. We are greatly indebted to Dr. Kanolt for the valuable assistance he has given us. The equivalent isohydrionic acid solutions that were subsequently prepared by the junior author gave confirmatory digestive results.

Bases.—We used ordinary bases of various types. Their names and formulas are appended:

Inorganic:
- Sodium carbonate, Na_2CO_3
- Potassium hydroxid, KOH
- Ammonium hydroxid, NH_4OH

Organic:
- Aliphatic—Tetra-ethyl ammonium hydroxid, $(C_2H_5)_4{\equiv}N{-}OH$
- Ethylene di-amin, $\begin{array}{l} CH_2{-}NH_2 \\ | \\ CH_2{-}NH_2 \end{array}$
- Tri-methyl amin, $(CH_3)_3{\equiv}N$
- Aromatic—Piperidin, $H_2C\left\langle \begin{array}{c} CH_2{-}CH_2 \\ \\ CH_2{-}CH_2 \end{array} \right\rangle NH$
- Conin, $H_2C\left\langle \begin{array}{c} CH_2{-}CH_2 \\ \\ CH_2{-}CH \end{array} \right\rangle NH$, with C_3H_7 attached to the CH
- Piperazin, $HN\left\langle \begin{array}{c} CH_2{-}CH_2 \\ \\ CH_2{-}CH_2 \end{array} \right\rangle NH$

[1] The data summarized in Cohn's *Indicators and Test Papers* were relied upon for accurate indications.

[2] The equinormal (isohydric) solutions were prepared in large volumes for the initial digestion experiments. They were also used later in the special acidalbumin study by Hawk and Gies (*loc. cit.*), in the work done by Loeb and Gies (*Arch. f. d. ges. Physiol.*, xciii, p. 246, 1902; also Gies and collaborators, *Biochemical Researches*, i, Reprint No. 29, 1903), and have since been employed in this work. Many of the earlier experiments have been repeated with newly prepared acid solutions; their results have invariably been confirmed.

Our solutions were made of the best obtainable samples of the bases indicated. The original ethylene di-amin was a 10 per cent. aqueous solution (Schering and Glatz) containing a small proportion of impurity, chiefly ammonium hydroxid. The tri-methyl amin product from which our solution was made was a 10 per cent. aqueous solution (Merck) containing some ammonium hydroxid. The remaining bases appeared to be practically pure. In all probability the recognized impurities had no marked effect on the general outcome of the experiments. See page 536.

The basic solutions employed in our work were of one general type—equidissociated (isohydroxidionic). They were prepared in three concentrations—each approximately equal in hydroxidion (OH′) content to that of a 0.25 per cent., 0.5 per cent., or 1.0 per cent. aqueous sodium carbonate solution.

As is well known an aqueous solution of sodium carbonate contains hydroxyl ions, which result from the hydrolytic action of the water on the carbonate with the formation apparently of small quantities of ionized sodium bi-carbonate and ionized sodium hydroxid, as is indicated below:

$$Na_2CO_3 + HOH \rightleftarrows Na^{\cdot}, OH' + Na^{\cdot}, HCO_3'$$

All the bases selected for use in this work undergo electrolytic dissociation, though in widely different degrees under similar conditions.

Shields[1] determined the concentration of hydroxyl ions in solutions of sodium carbonate of various strengths, by determining the speed with which such solutions saponified an ester. The following results were obtained by him:

Molecular Concentration of Na_2CO_3	Percentage Concentration of Na_2CO_3	Concentration of OH′: gram-mols per 1000 liters.	Hydrolytic Dissociation. Per cent.
0.1900	2.00	8.04	2.12
0.0940	1.00	5.96	3.17
0.0477	0.50	4.65	4.87
0.0238	0.25	3.38	7.10

The desired equivalent concentrations of the other bases employed in these experiments were determined by calculation with the aid of Shields's and Bredig's[2] data.

The desired product was made by dissolving the weighed quantity of base in the corresponding volume of water and determining the exact strength of the solution with $\frac{N}{10}$ or $\frac{N}{40}$ sulfuric acid. Titration was accomplished with the aid of congo red or phenolphthalein, and further addi-

[1] Shields, *Zeitschr. f. physikal. Chem.*, xii, p. 177, 1893.

[2] Bredig, *ibid.*, xiii, p. 289, 1894.

tions of water or base were made until exact equivalence with the standard was assured.[1]

ENZYME PREPARATIONS.—*Pepsin Products.*—Four preparations of pepsin were employed, which are designated in our summaries (page 502, *et seq.*) as follows:

"Pepsin—A." Merck's, 1:4000. Purchased, 1901.

"Pepsin—B." This preparation (1903) was a glycerol extract of gastric mucosa, prepared especially for this work as follows: Pig stomachs, shortly after their removal from recently slaughtered animals, were opened and the loosely adherent mucus removed with a dry towel. The mucous membrane was separated, spread on a glass plate, and peptic material obtained by scraping the surface of the membrane with a scalpel. The mucous matter thus separated was placed in a concentrated, aqueous 50 per cent. solution of glycerol, and extraction in it continued several days. The clear extract was then drawn off, strained, bottled, and used without further treatment. It was found to be strongly peptolytic.[2]

"Pepsin—C." Parke, Davis, and Co.'s, 1:3000. Purchased, 1903.

"Pepsin—D." Parke, Davis, and Co.'s, 1:2000. Purchased, 1905.

Trypsin Products.—Three preparations of trypsin were used, which are designated in our summaries (page 538, *et seq.*) as follows:

"Trypsin—A." Prepared as follows: Sheep pancreas was minced and thrown into a large excess of alcohol. The alcohol was renewed occasionally for several days. The residual tissue mass was then dried at room temperature. After all alcohol had disappeared 75 grams of the dry material were extracted in about 500 c.c. of a 0.1 per cent. aqueous solution of salicylic acid at 40° C. for about 10 hours. The filtered extract was poured into a large excess of alcohol. The precipitate thus obtained was strongly tryptic. The dry product was powdered and desiccated over sulfuric acid. It was quite hygroscopic but was kept dry in order to prevent inaccuracies in weighing.

"Trypsin—B." "Trypsin Merck." Purchased, 1906. This product was not appreciably hygroscopic.

"Trypsin—C." "Trypsin Merck." Purchased, 1906. Similar to trypsin "B" in gross aspects.

PROTEIN PRODUCTS.—Three different types of protein indicators were used: fibrin, elastin, and edestin. These proteins were selected because

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[1] Helpful facts in this connection and in relation to the concentration of hydrion in solutions of hydrochloric acid containing sulfuric acid (page 532) were given us by Prof. J. L. R. Morgan, for whose valued assistance it is a pleasure to render appreciative acknowledgment.

[2] This extract was prepared in May, 1903. Glycerol was the only preservative employed. The proteolytic power of the extract seems to be undiminished (November, 1906) and the extract itself appears to be only slightly altered. It is somewhat darker in color, and has acquired an odor resembling that of concentrated meat extract.

they can be prepared readily in large quantities and can be freed almost perfectly from adventitious matter, especially from dissociable salts.[1] They can easily be rendered anhydrous at 110° C., after preliminary treatment with alcohol and ether and drying at room temperature, without seriously affecting their digestibility in peptic or tryptic solutions. The light, loosely fibrous character of anhydrous fibrin or elastin particles make granular samples of these two proteins particularly well adapted for work of this kind. The light, white powder in which crystalline edestin may be prepared is also a very valuable indicator in proteolytic work. The convertibility of fibrin or edestin (in peptic or tryptic liquids) into proteinates ("albuminates") that are rendered insoluble in the main on neutralization of their solutions, makes quantitative determination of two general classes of the soluble digestive products convenient. Approximate estimation of the general zymolytic partition of these protein indicators is also rendered relatively easy, as a consequence. These three proteins were of further advantage in this work because they represent three fairly distinct grades of protein solubility in dilute acid or alkali, and exhibit similar well marked differences in resistance to proteolysis. (See pages 518–19.)

Fibrin.—Three preparations of fibrin were made as follows:

"Fibrin—A."[2] Fibrin, from ox blood, was finely minced in a hashing machine within an hour after its separation at the abattoir. It was kept in clean running water over night. In the morning it was again passed through a hashing machine and returned to clean running water until evening. By that time the pieces were almost perfectly exsanguinated. Persistently bloody particles were removed mechanically and discarded. The main mass of bloodless pieces was pressed in cheese cloth and transferred to 10 per cent. alcohol over night. Washing in alcohol was frequently repeated for several days. The strength of the alcohol was increased for each washing until absolute alcohol was used, after which the material was thoroughly washed free from alcohol with anhydrous ether. The product was freed from ether by evaporation at room temperature. The particles were light, white, and loosely fibrous. The fibrin was then further granulated in a pulverizer, after which treatment it consisted of very small particles that were fairly uniform in size. In this state samples of the material, as they were needed, were heated at 110° C. until all traces of moisture, alcohol, and ether were eliminated. Although the specially dried material was kept in well stoppered bottles, samples of it were subjected to repetitions of the desiccation process, in order to insure the absence of atmospheric moisture at the time the material was needed. This preparation was first used in 1901.

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1 A very important consideration, when the effects of ions are under investigation. See page 497.

2 This and similar designations are employed in our summaries, page 502, *et seq.*

"Fibrin—B" was made in 1902 by the method used in the preparation of "fibrin—A." In addition to the treatment accorded "fibrin—A", the original fibrin hash of this preparation was stirred in *boiling* water for about 5 minutes before treatment with alcohol was begun.

"Fibrin—C" was made in 1903 by exactly the same method as that used in the preparation of "fibrin—A."

Elastin.—The preparation of elastin used in this work was a finely granulated sample of one of the pure elastin products made and analyzed by Richards and Gies.[1] Samples were dried to constant weight at 110° C. before they were used.

Edestin.—Our preparation of edestin was made from hempseed by Osborne's method.[2] The light, white, crystalline powder was dried to constant weight at 110° C. before its employment.

Inorganic Contents of the Protein and Enzyme Products Employed.—That none of the digestive results summarized in this paper could have been appreciably modified by inorganic ions introduced with or formed from the protein or enzyme products used in the experiments is evident from an examination of the appended table, which gives the ash product from each protein[3] and enzyme preparation taken:

Amount of substance analyzed	Ash (expressed in milligrams).										
	Proteins.				Enzyme Products.						
	Fibrin.			Elastin	Pepsin.				Trypsin.		
Milligrams	A	B	C		A	B	C	D	A	B	C
1000	6.90	5.90	5.10	3.60	19.40	5.10	25.50	32.00	94.20	50.80	61.50
100	0.69	0.59	0.51	0.36	1.94	0.51	2.55	3.20	9.42	5.08	6.15
10	0.07	0.06	0.05	0.04	0.19	0.05	0.26	0.32	0.94	0.51	0.61

B—Digestive Procedure.

In all the digestion experiments the following general plan of procedure was followed:

Water Bath.—We used a circular, tin-plate, water bath nearly 7 inches deep and a foot in diameter, containing a loosely fitting double platform of zinc that could easily be turned or raised. The legs on which the double platform rested were 2 inches long, and consequently the lower shelf in the platform was separated from the bottom of the bath by a layer of water 2 inches deep. The double platform was open at the circumference. The slender perpendicular attachments between the lower and upper shelves of the movable platform were about 1½ inch long. The upper shelf had a dozen perforations, each of which was a little more

[1] Richards and Gies, *Amer. Journ. of Physiol.*, vii, p. 93, 1902; also Gies and collaborators, *Biochemical Researches*, i, Reprint No. 4, 1903.

[2] Osborne, *Amer. Chem. Journ.*, xiv, p. 28, 1893.

[3] All of our edestin preparation was inadvertently used up in another research before a determination of its inorganic matter could be made.

than 2 inches in diameter. The containers of the digestive mixtures fitted these perforations loosely and rested on the lower shelf of the platform, in which were many perforations half an inch in diameter.

The bottom of the bath was 7 inches from the table. Heat was supplied from a steady minute gas flame. The movable platform was intermittently raised and lowered during an experiment to favor rapid and equal distribution of the heat. No difficulty was experienced in keeping the temperature of the water practically constant.

CONTAINERS.—The digestive containers were heavy wide-mouthed glass bottles with ground glass stoppers. The bottles fitted the upper shelf perforations loosely. The bottles were nearly 6 inches high and had a capacity of 130 c.c. The level of the solutions in the containers was below that of the surrounding water. The width of the neck of each container made introduction of enzyme and protein particularly easy. There was also no difficulty whatever in completely removing digestive residues for gravimetric determination.

DIGESTIVE MIXTURES.—*Volume of Acid or Base.*—In every experiment the volume of each acidic or basic solution was 100 c.c. Immediately after this volume of the solution had been transferred to the digestive container, the latter was tightly stoppered. Up to the time that filtration was started the stopper of the container was removed twice; once, for the introduction of enzyme; again for the transferal of protein—each time only for a very brief period. Appreciable evaporation and attendant changes in degrees of concentration and ionization were thus entirely prevented until filtration was started.

The volume selected was such that the mixtures could be gently shaken without projecting liquid against the stopper and thus without causing temporary removals by capillarity from the main volume, with their attendant unequal influences on the digestive process.

Transference of Enzyme.—In experiments of this kind, in which comparative data are sought, it is obvious that the difficulties attending exactly equal distribution of enzyme in a series of solutions are particularly great. The activity of even a trace of enzyme is usually so marked that the greatest care was necessary in this connection, and every precaution was taken to prevent all but the very slight and insignificant errors that are entirely unavoidable in such work.

The enzyme was transferred to the digestive solution in either the dry state or in dissolved form. When a relatively large quantity of an enzyme product was used in each solution of a series, *e.g.*, 200 mgs., the amount was quickly and accurately obtained on a watch glass on a fine balance, and transferred to the solution through a small dry funnel from which the tube had been removed. By tilting the watch glass carefully over the funnel all but a few minute particles of the enzyme product slid collectively into the solution. The adherent particles could easily be brushed into the solution. Losses from "dusting" were inappreciable.

When particularly *small* quantities of any enzyme product were used in the solutions of a series, *e.g.*, 10 mgs. in each, it seemed probable that

even slight and unavoidable errors in weighing individual masses of 10 mgs. might be sufficient to introduce confusing variations in the digestive results. Consequently such small amounts as 10 mgs. of the enzyme preparation were introduced into the bottles of a series in minute volumes of a freshly prepared aqueous solution.[1] This solution was concentrated to such a degree that, as a rule, not more than 0.1 c.c. of it was required to give the desired quantity of enzyme. The enzyme solution was delivered from a buret or accurately calibrated pipet. Uniformity of measurement was insured for each series of experiments by the use of the same tube, by the removal of the same number of drops of enzyme-containing fluid for each member of the series, by the delivery of the fluid from a column that was kept practically the same in height, and by the maintenance of uniform external conditions about the orifice of the tube.

The enzyme was almost always transferred to the main solution a few minutes before the protein was added and after the desired temperature had been reached.

Protein Indicator.—In all but a few experiments we used for each digestive observation 1 gram of protein, dried to constant weight at 100–110° C.[2] Exactly 1 gram of the protein was quickly weighed on a fine balance and conveyed to the container by the method above described for the transferal of dry enzyme. The stoppered container was taken from the bath to the balance for the process, but was immediately returned to prevent appreciable fall of temperature. The time at which the protein was dropped into the solution was recorded as the beginning of the digestive period for that particular mixture.

The particles of the protein indicator were so nearly uniform in size that unavoidable initial inequalities of total surface exposure of the protein to the solution must have been too slight for any appreciable consequent effects on the digestive results.

Digestive Period.—The period of digestion varied for the different series but was exactly the same for each member of a series. During the continuance of an experiment the movable platform was occasionally raised and turned, and the mixtures were repeatedly shaken individually. In this process every effort was made to subject each mixture to exactly the same treatment.

Quantitative Determination of Digestive Products.—*Filtration* (removal of *undigested matter*).—As soon as the scheduled digestive period expired for the mixture that received the first sample of protein, filtration of that mixture was begun on a dry, weighed paper in readiness for the operation. Each mixture was removed from the bath for this purpose in the order of its original introduction. As a rule ordinary filtration was prompt enough to make the use of special pressure-apparatus

[1] Modification of concentration or degree of dissociation as a result of such dilution was not sufficient in any case to affect the results to any appreciable extent.

[2] The exceptions are referred to on pages 519 and 521.

unnecessary. Naturally, in the case of every mixture in a state of active zymolysis, digestion both of residue and of products in the filtrate also continued to progress, though with diminished intensity, *during* the filtration process. The expedition and regularity with which filtration was always conducted, however, rendered such continued digestion comparatively slight in every series and whatever its extent may have been in any experiment, it could never have been sufficiently variable in a series to have modified the proportionate results.

The residue obtained in the initial filtration process was thoroughly washed with water until the washings were entirely neutral. It was then dried to constant weight at 110° C. and weighed. The washing process was usually accomplished without delay.

Neutralization (precipitation of proteinate).—As a rule the amount of acidalbumin present in the digestive filtrates at a given time was determined. The method employed was that described by Hawk and Gies.[1] Neutralization was completed in the *hot* liquids. It was obviously impracticable to precipitate the proteinate before the mixture was filtered, but while the residue was being isolated on the filter paper, digestion in the accumulating filtrate continued, though with decreasing rapidity. The digestives periods for residue and proteinate (neutralization precipitate) could not be made the same therefore.

Our tables specify, for each of the series of digestions in which neutralization precipitates were obtained, the length of the main digestive period (before filtration was started) and the added time until neutralization of the filtrate for the precipitation of proteinate was accomplished. Both periods were of uniform length for the filtrates of a given series of experiments. The digestive period for the residue is obviously the first of the two periods referred to; that for the proteinate is, roughly speaking, the total time of the two periods. The figures for time in the summaries of this work (page 502, *et seq.*) represent the closest permissible approximations to the actual periods of time involved in the various operations. It is sufficient to know for a correct estimation of our results, that the departures from the absolute time for each operation in this connection were always insignificant and were practically the same for each member of a series. If it be inferred, because of certain inequalities in the recorded amounts of residue and dissolved products in each series, that significant quantitative errors have occurred to prevent greater uniformity of the digestive results it will be found on examination that obviation of the presumed experimental defects would have *strengthened* the evidence on which our conclusions are based. Our experimental errors must have been relatively trivial so far as possible prevention of quantitative uniformity among the digestive results was concerned. In no case could the prevention of any such slight and unavoidable departures have brought about greater similarity in the results for any series than in those actually recorded. More perfect methods would certainly have emphasized all our deductions from the results of this work.

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[1] Hawk and Gies, *loc. cit.*

In view of the fact that the amount of neutralization precipitate obtainable was frequently quite small, we used comparatively large proportions of each filtrate for the determination of the substance. The volume selected was usually 80 c.c.; occasionally it was not more than 50 c.c. As soon as the desired volume was obtainable it was transferred to a beaker and speedily neutralized. Appropriately dilute solutions of potassium hydroxid or sodium hydroxid were used for the peptic mixtures; of hydrochloric acid for the tryptic filtrates. When it was necessary to delay the process of *exact* neutralization a slight excess of the neutralizing reagent was added until the opposite reaction was attained, when the mixture was allowed to stand, until final neutralization could be effected, without danger of digestive continuance, because the enzyme was thus rendered inactive if it was not entirely destroyed.

After the filtrate had been neutralized exactly, or, if the reaction was allowed to remain slightly acid,[1] (when the maximum amount of proteinate appeared to be in sight), the flocculent mixture was set aside over night for sedimentation of the flakes. It was then filtered on a weighed paper; the precipitate was washed free from saline matter, then dried to constant weight at 110° C. and weighed.

Combined proteoses and peptones were estimated by difference. The sum of the weights of the residue and proteinate subtracted from the weight of the protein originally taken gave figures closely representing the combined amounts of proteoses and peptones. In several experiments (in which proteinate was not formed) a given proportion of the filtrate (10 c.c.) was neutralized and added to 350–500 c.c. of 98 per cent. alcohol for the precipitation of proteoses. The latter precipitates were filtered, washed with 95 per cent. alcohol, dried at 110° C., and weighed. The results in this connection were not important enough, however, to warrant the extra labor of incinerating the products for determination of the proportions of organic matter in them.

C—Quantitative Results of the Experiments.

I—Comparative Peptolysis.

Types of Acid Solutions Employed.

The fact that pepsin does not exhibit digestive power in the absence of a dissociable acid or acid compound implies a primary influence of the hydrogen ion in peptic proteolysis. It has frequently been observed that various acids are effective factors in peptolysis though in different degrees. We endeavored to ascertain whether the action of pepsin in a hydrionic solution is dependent upon only one type of ion, *i.e.*, hydrion ($H^{\cdot}$), or whether it is markedly influenced, also, by the associated anions (or molecules or both). Accordingly we studied peptic proteolysis in acid solutions of the following general types: (1) equi-

[1] Hawk and Gies, *loc. cit.*

percentage, (2) equi*molecular*, (3) equi*normal* (iso*hydric*), and (4) equi*dissociated* (iso*hydrionic*).

Summaries and Discussions of the Results.

The results of the peptolytic experiments are summarized in the following tables, (pages 502–536), where the descriptive headings make evident all the *special* experimental details of each series. The particular digestive results are considered in connection with each group of summaries. A discussion of the general results is undertaken on page 525.

Experiments 1-4. With Equipercentage Acid Solutions.

Conditions of the experiments. **All: Pepsin—D. Temperature—40° C.**

1 and 2. First series—with **fibrin** (A). Pepsin—10 mgs. (0.1 c.c. of a 10 % solution). Digestive periods—4 hours. Neutralization—1 hour after filtration was started. [In this and all other summaries, "R" signifies residue; "N.P.," neutralization precipitate; "P.P.," combined proteoses and peptones.]

Acid.				Weights of Products.			Relative Peptolysis.			Sequence.		
Nature.	Concentration.			R	N.P.	P.P.	R	N.P.	P.P.	R	N.P.	P.P.
	%	$\frac{mol}{x}$[1]	H˙ gram-atoms per 1000 liters	mgs	mgs	mgs	HCl product equals 10	100	100			
1.												
Hydrochloric	0.2	18	53[2]	130	241	629	10	100	100	2	1	2
Chloric	0.2	42	23	316	203	481	24	84	76	4	3	4
Nitric	0.2	32	31	299	209	492	23	87	78	3	2	3
Sulfuric	0.2	49	29	648	70	282	50	29	45	6	6	6
Phosphoric[3]	0.2	49	29	111	199	690	9	83	110	1	4	1
Arsenic[3]	0.2	71	22	818	24	158	63	10	25	7	7	7
Acetic	0.2	30	1	962	22	16	74	9	23	8	6	8
Oxalic	0.2	45	15	450	127	423	35	53	67	5	5	5
2.												
Hydrochloric	0.2	18	53	124	250	626	10	100	100	1	1	2
Chloric	0.2	42	23	335	194	471	27	78	75	4	2	4
Nitric	0.2	32	31	312	167	521	25	67	83	3	4	3
Sulfuric	0.2	49	29	670	40	290	54	16	46	6	6	6
Phosphoric	0.2	49	29	137	182	681	11	73	109	2	3	1
Arsenic	0.2	71	22	848	14	138	68	6	22	7	7	7
Acetic	0.2	30	1	978	1	21	79	0	3	8	8	8
Oxalic	0.2	45	15	485	140	375	39	56	60	5	5	5

[1] We have used the symbol $\frac{mol}{x}$ for the sake of convenience in our tables, to indicate *fractional* molecular concentrations. The figures in the columns underneath this device may be considered to occupy the position of x and to indicate, therefore, fractional molecular concentrations, such as $\frac{m}{18}$, $\frac{m}{42}$, etc., in the summary for experiment No. 1.

[2] Whole numbers are given in this and the other summaries under this head for the sake of convenience. The figures for H˙ concentration are not greatly affected by differences in temperature between 25° C. and 40° C.

[3] In calculating the hydrion concentrations of the solutions of phosphoric and arsenic

3 and 4. Second series—with elastin. Pepsin—400 mgs. (dry). Digestive periods—7 hours. Precipitation with alcohol—15 minutes after filtration was started.

Acid. Nature.	Acid. Concentration. %	Acid. Concentration. mol/x	Acid. Concentration. H· gram-atoms per 1000 liters	Weights of Products. R mgs	Weights of Products. P. P. Total mgs	Weights of Products. P. P. Precip'd mgs	Relative Peptolysis. R (HCl product equals 10)	Relative Peptolysis. P.P. Total (HCl product equals 100)	Relative Peptolysis. P.P. Precip'd (HCl product equals 100)	Sequence R	Sequence P.P. Total	Sequence P.P. Precip'd
3.												
Hydrochloric	0.2	18	53	273	727	—	10	100	—	1	1	—
Chloric	0.2	42	23	460	540	—	17	74	—	7	7	—
Nitric	0.2	32	31	426	574	—	16	79	—	5	5	—
Sulfuric	0.2	49	29	356	644	—	13	89	—	2	2	—
Phosphoric	0.2	49	29	380	620	—	14	85	—	3	3	—
Arsenic	0.2	71	22	456	544	—	17	75	—	6	6	—
Acetic	0.2	30	1	563	437	—	21	60	—	8	8	—
Oxalic	0.2	45	15	406	594	—	15	82	—	4	4	—
4.												
Hydrochloric	0.2	18	53	325	675	310	10	100	100	1	1	4
Chloric	0.2	42	23	434	566	310	13	84	100	7	7	5
Nitric	0.2	32	31	420	580	250	13	86	81	5	5	7
Sulfuric	0.2	49	29	334	666	460	10	99	148	2	2	1
Phosphoric	0.2	49	29	342	658	330	11	97	106	3	3	3
Arsenic	0.2	71	22	406	594	250	12	88	81	4	4	6
Acetic	0.2	30	1	570	430	210	18	64	68	8	8	8
Oxalic	0.2	45	15	423	577	400	13	85	129	6	6	2

Average digestive sequence (1–4):

Fibrin (1–2): *Hydrochloric* and phosphoric; nitric, chloric, *oxalic*, sulfuric, arsenic, *acetic*.

Elastin (3–4): *Hydrochloric;* sulfuric and phosphoric; arsenic and *oxalic;* nitric, chloric, *acetic*.

On the simple solubility of the protein products in the equipercentage acid solutions used in exp. 1–4. Elastin is only slightly soluble in

acids, it was assumed that these acids undergo electrolytic dissociation in harmony with the following equations:

$$H_3PO_4 \rightleftarrows 3H^{\cdot} + PO_4'''$$

$$H_3AsO_4 \rightleftarrows 3H^{\cdot} + AsO_4'''$$

Complete dissociation as here indicated takes place only in very dilute solutions. Under ordinary circumstances, solutions of phosphoric acid contain the following ions: $H^{\cdot}$, H_2PO_4', HPO_4'' and PO_4'''. Analogous ions are present in ordinary arsenic acid solutions. There are no exact methods of determining the concentrations of any of these ions in solutions of the two acids indicated. The figures in our summaries for the hydrion concentrations of our solutions of phosphoric acid and arsenic acid are only approximate, therefore. A consideration of the various facts in the cases mentioned convinces us that the differences between the figures given in our tables and the true values, were they obtainable, cannot be sufficient to affect our general conclusions.

dilute acid at ordinary temperatures.[1] Our digestive results with elastin (experiments 3 and 4) could not have been materially affected by the slight simple solvent action exerted by the acids alone. The same may be said of our data for fibrin (experiments 1 and 2), as the results in the following résumé will show.

Special experiment to determine the solvent action on fibrin of the equipercentage acids used in experiments 1–4. Conditions of the experiment: Fibrin—A; amount—1 gram. Temperature—40° C. Period of exposure to the action of the acids—4 hours. Neutralization—1 hour after filtration was started.

Acid.	Hydrochloric.	Chloric.	Nitric.	Sulfuric.	Phosphoric.	Arsenic.	Acetic.	Oxalic.
Fibrin residue (mgs.)	961	981	976	992	980	989	956	982
Neutralization precipitate (mgs.)	14	10	6	5	17	7	4	6
Substance unrecovered (mgs.)	25	9	18	3	3	4	40	12

These results made it evident that no corrections of our digestive results were necessary on account of the slight, simple solubility of the proteins in the acids themselves. If such corrections had been made, however, in every instance, none of the conclusions drawn from our results would have been different.

Special digestive results of the experiments with equipercentage acid solutions. In experiments 1–4 wide quantitative digestive differences were observed in each group. The proteolytic sequence was different for each protein, although both proteins digested most in hydrochloric acid and phosphoric acid, and least in acetic acid. Peptolysis was clearly unequal.

Digestion of fibrin was particularly tardy in the sulfuric, arsenic, and acetic acid solutions. Elastin was not rapidly digested in any of the solutions, but was transformed somewhat more slowly in the acetic acid solution than in the others. There was a particularly marked difference in the peptolytic efficiencies of sulfuric, arsenic, nitric, and chloric acids in the digestion of the two protein products employed; elastin digesting relatively more readily than fibrin in sulfuric and arsenic acids but less readily in nitric and chloric acids. Oxalic acid was about the same in each series in effecting a medium rate and extent of peptolysis. Acetic acid was also the same in each series in showing the least tendency to co-operate with the pepsin. In each series peptolysis

[1] Richards and Gies, *loc. cit.*, p. 104.

was relatively speedy and complete in the hydrochloric and phosphoric acid solutions.

A very conspicuous difference in the digestion of fibrin was manifested in the solutions of phosphoric and sulfuric acids, which were practically the same in percentage, molecular, and hydrion concentrations. This disagreement was seemingly due to divergent effects of the different anions (and probably also the molecules) involved. The disagreements throughout each series were doubtless due largely to these same influences. In the digestion of elastin no such special quantitative disagreement between the influence of phosphoric and sulfuric acids was observed. Sulfuric acid like phosphoric acid was almost as efficient as hydrochloric acid in the digestion of elastin.

Additional data which show that the foregoing results are not peculiar to the particular percentage concentration employed are given on page 526.

Experiments 5–8. With Equimolecular Acid Solutions.

Conditions of the experiments. All: Pepsin—D. Temperature—40° C.

5 and 6. First series—with fibrin (A). Pepsin—10 mgs. (0.1 c.c. of a 10 % solution.) Digestive periods—4 hours. Neutralization—1 hour after filtration was started.

Acid: Nature	Acid: Concentration: mol/x	Acid: Concentration: %	Acid: Concentration: H˙ gram-atoms per 1000 liters	Weights of Products: R (mgs)	Weights of Products: N P (mgs)	Weights of Products: P P (mgs)	Relative Peptolysis (HCl product equals): R (10)	Relative Peptolysis (HCl product equals): N P (100)	Relative Peptolysis (HCl product equals): P P (100)	Sequence: R	Sequence: N.P.	Sequence: P.P.
5.												
Hydrochloric	30	0.12	32	147	278	575	10	100	100	2	1	2
Chloric	30	0.28	32	285	210	505	19	76	88	3	3	4
Nitric	30	0.21	32	312	200	488	21	72	85	5	4	5
Sulfuric	30	0.33	43	650	60	290	44	22	50	7	7	7
Phosphoric	30	0.33	40	105	213	682	7	77	119	1	2	1
Arsenic	30	0.47	39	383	168	449	26	60	78	6	6	6
Acetic	30	0.20	1	951	15	34	65	5	6	8	8	8
Oxalic	30	0.30	22	295	185	520	20	67	90	4	5	3
6.												
Hydrochloric	30	0.12	32	126	245	629	10	100	100	2	2	2
Chloric	30	0.28	32	252	191	557	20	78	89	4	3	4
Nitric	30	0.21	32	299	277	424	24	113	67	5	1	6
Sulfuric	30	0.33	43	619	56	325	49	23	52	7	7	7
Phosphoric	30	0.33	40	72	167	761	6	68	121	1	4	1
Arsenic	30	0.47	39	348	111	541	28	45	86	6	6	5
Acetic	30	0.20	1	947	4	49	75	2	8	8	8	8
Oxalic	30	0.30	22	248	126	626	20	51	100	3	5	3

7 and 8. Second series—with **elastin.** Pepsin—400 mgs. (dry). Digestive periods—7 hours. Precipitation with alcohol—15 minutes after filtration was started.

Acid.				Weights of Products.			Relative Peptolysis.			Sequence.		
Nature.	Concentration.			R	P.P.		R	P.P.		R	P.P.	
	mol/x	%	H· gram-atoms per 1000 liters		Total	Precip'd		Total	Precip'd		Total	Precip'd
				mgs	mgs	mgs	HCl product equals 10	100	100			
7.												
Hydrochloric	30	0.12	32	403	597	—	10	100	—	4	4	—
Chloric	30	0.28	32	459	541	—	11	91	—	6	6	—
Nitric	30	0.21	32	467	533	—	12	89	—	7	7	—
Sulfuric	30	0.33	43	336	664	—	8	111	—	1	1	—
Phosphoric	30	0.33	40	365	635	—	9	106	—	2	2	—
Arsenic	30	0.47	39	397	603	—	10	101	—	3	3	—
Acetic	30	0.20	1	613	387	—	15	65	—	8	8	—
Oxalic	30	0.30	22	408	592	—	10	99	—	5	5	—
8.												
Hydrochloric	30	0.12	32	340	660	330	10	100	100	1	1	3
Chloric	30	0.28	32	444	556	220	13	84	67	7	7	8
Nitric	30	0.21	32	411	589	290	12	89	88	6	6	5
Sulfuric	30	0.33	43	347	653	710	10	99	215	2	2	1
Phosphoric	30	0.33	40	365	635	320	11	96	97	3	3	4
Arsenic	30	0.47	39	379	621	280	11	94	85	4	4	6
Acetic	30	0.20	1	578	422	270	17	64	82	8	8	7
Oxalic	30	0.30	22	397	603	620	12	91	188	5	5	2

Average digestive sequence (5–8):

Fibrin (5–6): Phosphoric and hydrochloric; chloric and oxalic; nitric, arsenic, sulfuric, and *acetic*.

Elastin (7–8): Sulfuric, phosphoric, and hydrochloric; arsenic, oxalic, nitric, and chloric; *acetic*.

Special digestive results of the experiments with the equimolecular acid solutions. Most of the foregoing statements about the data of our experiments (1–4) with the equipercentage acid solutions are true of the results of the experiments (5–8) with the equimolecular solutions. In the latter solutions the concentration of the hydrochloric acid was less than in the solutions of experiments 1–4; of the nitric acid and acetic acid solutions the concentrations were practically the same as before; and in the remaining solutions the strength of the particular acid was somewhat greater than in the first four experiments. *Simple solubility effects were practically the same as for experiments 1-4.*

The digestive conditions of each type of experiment with the

equipercentage solutions were duplicated in those with the equimolecular mediums. On comparing the tabulated data for both kinds of solutions it is obvious that the quantitative and sequential results recorded in the summaries, especially in connection with fibrin, varied, to some extent at least, with the changes of hydrion (H˙) concentration. The more vigorous digestions of fibrin, for example, in the arsenic acid and oxalic acid solutions of $\frac{M}{30}$ strength illustrate the beneficial effects of increasing the concentration of hydrion. Digestion in the acids of unchanged concentration, *i. e.*, nitric and acetic acids was practically the same for each protein. In the hydrochloric acid solutions of experiments 5–8, proteolysis was only slightly reduced although the concentration of hydrogen cations was greatly diminished, as compared with that of the solutions used in experiments 1–4. The extreme quantitative differences that were noted among the results of the experiments in the equipercentage acid solutions were duplicated in a measure in experiments 5–8.

Additional results, obtained at different planes of molecular strength and which confirm the observations of experiments 5–8 are exhibited on page 527.

On the simple solubility of fibrin in the equinormal (isohydric) acid solutions used in experiments 9a–18. Some of the experiments in the isohydric acid solutions were among our first. At an early stage in our work we ascertained that practically all of the fibrin was recoverable from the isohydric acid solutions when pepsin was absent. We found that the amounts of neutralization precipitate were comparatively slight; that they were least in the strongest acid solutions employed, apparently because of the greater solvent action of the salts formed on neutralization.[1] The following results of some special experiments in this connection illustrate the data upon which the above conclusions rest:

A. Fibrin (B)—1 gram. Temperature—40° C. Period of exposure to the acids (100 c.c.)—4 hours.

Acid.	Weight of Recovered Substance. Gram.
Nitric, $\frac{M}{10}$	1.012
Arsenic, $\frac{M}{10}$	1.017

[1] Hawk and Gies, *loc. cit.*, p. 487.

B. Acid—hydrochloric (100 c.c.) Fibrin (A)—1 gram. Temperature—40° C. Period of exposure to the acid—1½ hour. Neutralization—1 hour after filtration was started.

Concentration	$\frac{M}{10}$	$\frac{M}{20}$	$\frac{M}{10}$
Neutralization precipitate (mgs.)	32	26	12

On the swelling of fibrin and edestin in the equinormal (isohydric) acid solutions. The bloating effects of acids on particles of fibrin and other proteins is well known. We have made note of such expansive results in these experiments. The following statements apply especially to our data in this connection for the isohydric solutions.

The *elastin* particles did not increase in bulk perceptibly in any of the acids with which they were treated. The *edestin* particles were promptly swollen by four of the acids in which there were digested, apparently in the order, and proportional to the extent, of their hydration in the same acids, as follows: hydrochloric, chloric, nitric, and oxalic. It required nearly 4 hours' contact with sulfuric acid for the edestin particles to show perceptibly a swollen aspect.

The most decided effects in this connection were exhibited by fibrins A and C (*unboiled* products). Swelling was usually rapid and pronounced in each of the acids except citric, sulfuric, and acetic. Even under the most favorable conditions of concentration of both acid and enzyme, and during the longest periods, in these experiments, sulfuric acid and acetic acid rarely gave any evidence whatever of a bloating influence on the fibrin particles. Under favorable conditions, however, citric acid always caused decided swelling. Even in the experiments in which fibrin was markedly digested in sulfuric acid or acetic acid, enlargement of the fibrin particles did not appear to precede hydration. Boiled fibrin (B) was more resistant to the expanding influence, even in hydrochloric acid, than the unboiled fibrin.

Swelling of the fibrin was always prompt in the acids in which digestion occurred rapidly. In some of the experiments, notably in 9a and 10, swelling was most decided in arsenic acid. In others, swelling was usually as decided in arsenic as in hydro-

chloric acid, but not more so. In experiments 9a and 10 the amounts of enzyme present were relatively large, the periods of digestion were proportionately long and peptolysis in nearly all the acids of each series was comparatively extended.

It seems probable that the anion of the acid is an important factor in bringing about an increased bulk of the fibrin particles. That there is a relation of some kind between the swelling and peptolysis of fibrin (and other proteins similarly swollen by comparatively dilute acid) has also been suggested by our results. There is probably some significance in the fact that the elastin particles, which did not increase in bulk in any of our acid solutions, were digested quite as well in sulfuric acid, which is conspicuously devoid of a swelling effect on fibrin, as in any other, and almost as well in acetic acid, which has even less bloating influence than sulfuric acid on fibrin.

In a general way promptness and extent (to the maximum) of the swelling process were exhibited in our isohydric acid solutions in approximately the following sequence: arsenic, hydrochloric, chloric, nitric, phosphoric, hydriodic, oxalic, lactic, tartaric, citric, sulfuric, and acetic. It is possible that this sequence is ordered by the digestion that begins about the time or perhaps before the swelling manifests itself. The amount of enzyme present seems to be a matter of importance in this connection. See remarks on page 522.

Special digestive results of the experiments with the equinormal (isohydric) acid solutions. In the experiments with equipercentage and equimolecular acid solutions the conditions for each group of experiments were kept uniform in order particularly to insure definite digestive results at the two different acid concentrations employed with each protein. In experiments 9a to 16 inclusive the conditions were varied, however, with special reference to the concentrations of acid and enzyme and the length of digestive period. The following facts were clearly established for fibrin in this connection:

The *greater* the active amount of pepsin and the *longer* the period of digestion of *fibrin* in equinormal acid solutions, the more nearly uniform the ultimate quantitative results became.

Even under the *most favorable circumstances* for the attainment of quantitative equality of peptolysis in all respects in such acid

solutions, however, digestion in slightly dissociable acids such as acetic and citric ("weak acids") was relatively slow and incomplete. The acids in which, under the most favorable uniform conditions, peptolysis of fibrin tended to be the speediest and most complete, were in general, those that dissociate to the highest degree ("strong acids").

The greatest peptolytic disparities were shown in the experiments in which comparatively small amounts of pepsin were used (11–14). If peptolysis is stopped in such solutions before it approximates completeness in any member of a group, perhaps the most definite expressions of the relative co-operative activities of pepsin and the acids are afforded.

Experiments 9a–18. With Equinormal (Isohydric) Acid Solutions.

Conditions of the experiments. **All: Temperature—40° C.** *9a—14. First series.* **Fibrin**—A. Pepsin—A; amounts—different. Digestive periods—4 hours. Neutralization—1 hour after filtration was started.

Acid.				Weights of Products.			Relative Peptolysis.			Sequence.		
Nature.	Concentration			R	N.P.	P.P.	R	N.P.	P.P.	R	N.P.	P.P.
	$\frac{mol}{x}$	%	H˙ gram-atoms per 1000 liters	mgs	mgs	mgs	HCl product equals 10	100	100			
9 a. Pepsin—100 mgs. (0.4 c.c. of a 25% solution).												
Hydrochloric	10	0.36	94	35	40	925	10	100	160	1	4	1
Chloric	10	0.84	93	58	81	861	17	203	93	4	1	6
Nitric	10	0.63	93	74	62	864	21	155	93	5	2	5
Sulfuric	20	0.49	61	122	55	823	35	138	89	7	3	7
Phosphoric	30	0.33	40	40	36	924	11	90	100	2	5	2
Arsenic	30	0.47	39	97	25	878	28	63	95	6	7	4
Lactic	10	0.90	4	252	12	736	72	30	80	9	9	9
Acetic	10	0.60	1	819	2	179	234	5	19	11	11	11
Oxalic[1]	20	0.45	29	47	32	921	13	80	100	3	6	3
Tartaric	20	0.75	6	204	14	782	58	35	85	8	8	8
Citric	30	0.64	5	347	9	644	99	22	70	10	10	10

[1]The figure for hydrion concentration of $\frac{m}{20}$ oxalic acid was obtained by extrapolation, since no values for conductivity at that dilution have been determined. The value given in our tables, though somewhat uncertain, is certainly closely approximative of the truth in the matter.

Acid.				Weights of Products			Relative Peptolysis			Sequence		
	Concentration			R	N P	P P	R	N P	P P			
			H˙				HCl product equals					
Nature.	mol/x	%	gram-atoms per 1000 liters	mgs	mgs	mgs	10	100	100	R	N P	P P.
9 b. Pepsin—200 mgs. (0.8 c c. of a 25% solution).												
Acetic	10	0 60	1	728	5	267	—	—	—	—	—	—
10. Pepsin—50 mgs. (0.2 c.c. of a 25% solution) Compare with 16.												
Hydrochloric	10	0 36	94	35	70	895	10	100	100	1	2	3
Chloric	10	0 84	93	61	75	864	17	107	97	4	1	5
Nitric	10	0 63	93	86	68	846	25	97	95	6	3	6
Sulfuric	20	0 49	61	138	56	806	39	80	90	7	4	8
Phosphoric	30	0 33	40	36	48	916	10	69	102	2	6	1
Arsenic	30	0 47	39	68	44	888	19	63	99	5	7	4
Lactic	10	0 90	4	199	22	779	57	31	87	9	8	9
Acetic	10	0 60	1	876	3	121	250	4	14	11	11	11
Oxalic	20	0 45	29	39	50	911	11	71	102	3	5	2
Tartaric	20	0 75	6	152	18	830	43	26	93	8	9	7
Citric	30	0 64	5	399	10	591	114	14	66	10	10	10
11. Pepsin—10 mgs. (0.1 c.c. of a 10% solution). Compare with 15.												
Hydrochloric	20	0 18	48	33	64	903	10	100	100	1	5	1
Hydriodic	20	0 64	49	57	93	850	17	145	94	4	1	5
Nitric	20	0 32	48	45	87	868	14	136	96	3	2	3
Sulfuric	40	0 25	34	258	57	685	78	89	76	6	6	6
Phosphoric	60	0 16	25	42	56	902	13	88	100	2	7	2
Arsenic	60	0 24	25	272	77	651	82	120	72	7	3	7
Acetic	20	0 30	1	939	4	57	284	6	6	8	8	8
Oxalic	40	0 23	17	69	69	862	21	108	95	5	4	4
12. Pepsin—5 mgs. (0.1 c.c. of a 5% solution).												
Hydrochloric	40	0 09	24	40	201	759	10	100	100	1	2	1
Hydriodic	40	0 32	25	143	103	749	37	51	99	3	3	2
Chloric	40	0 21	24	110	244	646	28	121	85	2	1	3
Arsenic	120	0 12	16	830	8	162	208	4	21	4	4	4
Lactic	40	0 23	2	938	4	58	235	2	8	5	5	5
Citric	120	0 16	2	967	0	33	242	0	4	6	6	6
13 a. Pepsin—4 mgs. (0 2 c c. of a 2% solution)												
Hydrochloric	40	0 09	24	49	219	732	10	100	100	1	1	1
Nitric	40	0.16	24	98	194	708	20	89	97	3	3	2
Sulfuric	80	0.12	19	509	83	408	104	38	56	4	4	4
Phosphoric	120	0 08	16	82	213	705	17	97	96	2	2	3
Acetic	40	0 15	1	994	1	5	203	0	1	6	6	6
Oxalic	80	0 11	9	628	39	333	128	18	46	5	5	5
13 b. Pepsin—8 mgs. (0.4 c.c. of a 2% solution).												
Hydrochloric	40	0 09	24	48	88	864	—	—	—	—	—	—
14 a. Pepsin—1.6 mg. (0.2 c.c. of an 0 8% solution).												
Hydrochloric	40	0 09	24	71	248	681	10	100	100	1	1	1
Nitric	40	0.16	24	169	238	593	24	96	87	3	3	3
Sulfuric	80	0.12	19	644	84	272	91	34	40	4	4	4
Phosphoric	120	0 08	16	127	243	630	18	98	93	2	2	2
Acetic	40	0 15	1	984	2	14	139	1	2	6	6	6
Oxalic	80	0 11	9	723	69	208	102	28	31	5	5	5
14 b. Pepsin—3.2 mgs. (0.4 c c. of an 0.8% solution).												
Hydrochloric	20	0 18	48	57	112	831	—	—	—	—	—	—

15–16. Second series. **Fibrin**—A or B. Pepsin—A; amounts—different. Digestive periods—different. Neutralization—1 hour after filtration was started.

Acid.				Weights of Products.			Relative Peptolysis.			Sequence.		
	Concentration.			R	N.P.	P.P.	R	N.P.	P.P.			
			H˙				HCl product equals					
Nature.	mol/x	%	gram-atoms per 1000 liters	mgs	mgs	mgs	10	100	100	R	N.P.	P.P.

15. Pepsin—10 mgs. (0.1 c.c. of a 10% solution). Fibrin—A. Digestive period—2 hours. Compare with 11.

Nature	mol/x	%	H˙	R	N.P.	P.P.	R	N.P.	P.P.	R	N.P.	P.P.
Hydrochloric	20	0.18	48	74	256	670	10	100	100	1	1	3
Chloric	20	0.42	48	139	136	725	19	53	108	3	3	2
Nitric	20	0.32	48	212	136	652	29	53	97	4	4	4
Sulfuric	40	0.25	34	628	64	308	85	25	46	6	6	6
Phosphoric	60	0.16	25	92	180	728	12	70	109	2	2	1
Arsenic	60	0.24	25	670	60	270	91	23	40	7	7	7
Lactic	20	0.45	3	830	12	158	112	5	24	8	8	8
Oxalic	40	0.23	17	301	128	571	41	50	85	5	5	5
Citric	60	0.32	3	954	1	45	129	0	7	9	9	9

16. Pepsin—50 mgs. (0.2 c.c. of a 25% solution). Fibrin—B. Digestive period—4 hours. Compare with 10.

Nature	mol/x	%	H˙	R	N.P.	P.P.	R	N.P.	P.P.	R	N.P.	P.P.
Hydrochloric	10	0.36	94	64	63	873	10	100	100	2	1	2
Chloric	10	0.84	93	199	36	765	31	57	88	4	4	4
Nitric	10	0.63	93	255	26	719	40	41	82	5	6	5
Sulfuric	20	0.49	61	507	8	485	79	13	56	8	10	8
Phosphoric	30	0.33	40	49	43	908	8	68	104	1	3	1
Arsenic	30	0.47	39	268	30	702	42	48	80	6	5	6
Lactic	10	0.90	4	650	14	336	102	22	38	9	9	9
Acetic	10	0.60	1	951	2	47	149	3	5	11	11	11
Oxalic	20	0.45	29	99	54	847	16	86	97	3	2	3
Tartaric	20	0.75	6	484	20	496	76	32	57	7	7	7
Citric	30	0.64	5	697	15	288	109	24	33	10	8	10

17–18. Third series. **Elastin.** Pepsin (D)—400 mgs. (dry). Digestive periods—7 hours.

Acid.				Weights of Products.		Relative Peptolysis.		Sequence.	
	Concentration.			R	P.P.	R	P.P.		
			H˙			HCl product equals			
Nature.	mol/x	%	gram-atoms per 1000 liters	mgs	mgs	10	100	R	P.P.

17.

Nature	mol/x	%	H˙	R	P.P.	R	P.P.	R	P.P.
Hydrochloric	10	0.36	94	444	556	10	100	4	4
Sulfuric	20	0.49	61	428	572	10	103	1	1
Phosphoric	30	0.33	40	430	570	10	103	2	2
Acetic	10	0.60	1	537	463	12	83	5	5
Oxalic	20	0.45	29	441	559	10	101	3	3

18. Repetition of experiment 17.

Nature	mol/x	%	H˙	R	P.P.	R	P.P.	R	P.P.
Hydrochloric	10	0.36	94	469	531	10	100	4	4
Sulfuric	20	0.49	61	428	572	9	108	1	1
Phosphoric	30	0.33	40	459	541	10	102	3	3
Acetic	10	0.60	1	548	452	12	85	5	5
Oxalic	20	0.45	29	452	548	10	103	2	2

The best digestive results for *fibrin* in the isohydric mediums were usually obtained in the hydrochloric acid solutions. Phosphoric acid was almost equally favorable. Digestion in sulfuric acid solutions was comparatively poor; no better than in solutions of arsenic acid. Oxalic acid was more helpful than either sulfuric acid or arsenic acid, and was practically equal to chloric, nitric, and hydriodic acids in supporting peptolysis. Chloric, nitric, and hydriodic acids stood next to hydrochloric and phosphoric acids in power to set up digestion. Oxalic acid was more favorable than any of the other organic acids. Proteolysis was always less pronounced in acetic acid than in any other solution. Tartaric, lactic, and citric acids, in the order named, occupied positions between oxalic and acetic acids in power to sustain peptolysis.

The *average* order of peptolytic efficiency (on *fibrin*) of the isohydric acid solutions was the following: Hydrochloric, phosphoric, chloric, nitric, hydriodic, oxalic, arsenic, sulfuric, tartaric, lactic, citric, acetic. This sequence was not the order of dissociation.

Absolute and relative rate and extent of peptolysis of fibrin in a given series of these acid solutions were markedly determined by the concentration of the enzyme. Thus, when considerable pepsin was present in the isohydric medium, peptolysis in the oxalic acid solutions, for example, was almost equal in extent to that in hydrochloric acid and in phosphoric acid. In the series in which only relatively slight proportions of pepsin were present, proteolysis in the oxalic acid solutions was no more extended than in the sulfuric acid solutions.

In the digestion of *elastin* (17–18) the results in this connection, as in previous ones, were more nearly uniform than for fibrin. Although elastin was digested much more slowly than fibrin, the degree of transformation into soluble products, even in acetic acid, was not far behind that which occurred in the acid solution of greatest efficiency—sulfuric acid.

The average sequence exhibited in the digestion of elastin in the isohydric solutions was the following: sulfuric, phosphoric, oxalic, and hydrochloric; acetic. The differences on which this sequence is ordered are very slight, especially for the first four acids named.

The inequality between fibrin and elastin in responsiveness to the action of pepsin is more evident in these particular experiments than in any others reported in this paper. As before, digestion of the elastin, in contrast with that of fibrin, was relatively pronounced in the sulfuric acid solutions. The difference between fibrin and elastin in their relative degrees of digestibility in acetic acid is especially noteworthy, in this connection also.

That the *rates* of digestion in all these experiments were always unequal in a series was very evident to us. Even when the final results exhibited fairly close equality, the rates of progress were distinctly unequal. Such notes as the following, quoted directly from our records, accompany practically all our results:

Exp. 15 "End of 1st hour —Digestion rapid though unequally so in HCl, H_3PO_4, HNO_3, and $HClO_3$; sequence as indicated. Distinct though gradual in oxalic, H_3AsO_4 and lactic, and seemingly that order. Very slight in H_2SO_4 and citric, though lack of swelling in these makes judgment uncertain."

These experiments (9a—18), show that, under uniform digestive conditions in a series, peptolysis of fibrin as well as of elastin is unequal in *isohydric* acid solutions. That extent and rate of peptolysis in isohydric solutions of acids are determined by a number of factors is evident. Among the conditioning influences exhibited in these particular experiments were character, state, and concentration of the acid, concentration of the enzyme, duration of the digestive period, and character of the protein indicator. Elastin behaved differently from fibrin. Fibrin that had been *coagulated* by heat (B) was not only more resistant to proteolysis than unheated fibrin (A), but its resistance to pepsin in the various acid solutions was irrelative. (Compare with experiments 10 and 16).

On the simple solubility of fibrin in the equidissociated (isohydrionic) acid solutions used in experiments 19-45. That our results with the isohydrionic acid solutions were not materially affected by the solvent action of the acids on the proteins in the absence of pepsin is apparent from the following data of some special experiments with fibrin in this connection:

A. Isohydrionic acids, used in experiments 19-39 (100 c.c.).

Fibrin (B)—1 gram. Temperature—25° C. Period of exposure to the acids—9 hours (!) Neutralization—30 minutes after filtration was started.

Acid.	Hydrochloric.	Chloric.	Nitric.	Sulfuric.	Oxalic.
Residue (mgs.)...........	1005	1029	1014	1014	1017
Neutralization precipitate (mgs.)...............	—	3	—	—	4

The amounts of neutralization precipitates in the chlorid, nitrate, and sulfate solutions were too slight for gravimetric determination.

B. Same acids as in the preceding experiment (100 c.c.). Fibrin (C)—1 gram. Temperature—25°C. Period of exposure to the acids—5½ hours. Neutralization—30 minutes after filtration was started.

Acid.	Hydrochloric.	Chloric.	Nitric.	Sulfuric.	Oxalic.
Residue (mgs.)...........	1009	1008	1012	1007	1003
Neutralization precipitate (mgs.)...............	4	4	2	1	7

Experiments 19–45. With Equidissociated (Isohydrionic) Acid Solutions.

19–39. First group of acid solutions—Hydrion ($H^{\cdot}$) concentration equal to that of a 0.19 % hydrochloric acid solution (at 25° C.).

The names of the acids and data bearing on their concentrations are summarized below:

Acid	$H^{\cdot}$: gram-atoms per 1000 liters	$\frac{\text{mol}}{x}$	%
Hydrochloric	50	19	0.19
Chloric	50	19	0.44
Nitric	50	19	0.33
Sulfuric	50	26	0.38
Oxalic	50	12	0.77

Conditions of the experiments. Each series different in one or more respects from all the others.

19–21. First series. Pepsin—A; amounts—different. Fibrin—C. Temperature—25° C. Digestive periods—different. Neutralization—30 minutes after filtration was started.

Acid.	Weights of Products.			Relative Peptolysis.			Sequence.		
Nature	R	N.P.	P.P.	R	N.P.	P.P.	R	N.P.	P.P.
				HCl product equals					
	mgs	mgs	mgs	10	100	100			

19. Pepsin—300 mgs.(dry). . Digestive period—4 hours.

Hydrochloric	18	Trace	982	10	—	100	1	—	1
Chloric	19	"	981	11	—	100	3	—	3
Nitric	21	"	979	12	—	100	4	—	4
Sulfuric	37	"	963	21	—	98	5	—	5
Oxalic	19	"	981	11	—	100	2	—	2

20. Pepsin—200 mgs.(dry). Digestive period—3 hours.

Hydrochloric	14	14	972	10	100	100	1	1	2
Chloric	17	8	975	12	57	100	3	4	1
Nitric	22	11	967	16	79	100	4	3	4
Sulfuric	104	8	888	74	57	91	5	5	5
Oxalic	16	12	972	11	86	100	2	2	3

21. Pepsin–100 mgs.(0.2 c.c. of a 50% solution). Digestive period—2 hours.

Hydrochloric	17	10	973	10	100	100	1	5	1
Chloric	30	17	953	18	170	98	3	3	3
Nitric	46	18	936	27	180	96	4	2	4
Sulfuric	268	29	703	158	290	72	5	1	5
Oxalic	20	13	967	12	130	99	2	4	2

22–25. Second series. Pepsin—A; amounts—different. Fibrin—C. Temperature—25° C. Digestive periods—1 hour. Neutralization—30 minutes after filtration was started.

22. *Pepsin—50 mgs. (0.1 c.c. of a 50% solution).*

Hydrochloric	36	136	828	10	100	100	1	1	1
Chloric	153	101	746	42	74	90	3	4	3
Nitric	171	113	716	47	83	86	4	3	4
Sulfuric	552	31	417	153	23	50	5	5	5
Oxalic	94	118	788	26	87	95	2	2	2

23. Pepsin—10 mgs.(0.1 c.c. of a 10% solution). Compare with 26.

Hydrochloric	78	243	679	10	100	100	1	1	1
Chloric	305	137	558	39	56	82	3	3	3
Nitric	394	81	525	51	33	77	4	4	4
Sulfuric	779	10	211	100	4	31	5	5	5
Oxalic	224	139	637	29	57	94	2	2	2

24. Pepsin—5 mgs.(0.05 c.c. of a 10% solution). Compare with 32.

Hydrochloric	168	264	568	10	100	100	1	1	2
Chloric	400	151	449	24	57	79	3	2	3
Nitric	486	99	415	29	37	73	4	4	4
Sulfuric	789	12	199	41	5	35	5	5	5
Oxalic	281	138	581	17	52	102	2	3	1

Acid.	Weights of Products.			Relative Peptolysis.			Sequence.		
Nature	R.	N.P.	P.P.	R	N.P.	P.P.	R	N.P.	P.P.
				HCl product equals					
	mgs	mgs	mgs	10	100	100			

25. Pepsin—2.5 mgs. (0.05 c.c. of a 5% solution).

Hydrochloric	229	312	459	10	100	100	1	1	1
Chloric	494	162	344	22	52	75	3	3	3
Nitric	637	87	276	28	28	60	4	4	4
Sulfuric	851	8	141	37	3	31	5	5	5
Oxalic	411	177	412	18	57	90	2	2	2

26–30 Third series. Pepsin—different preparations (A or D); amounts—different. Fibrin—different preparations (A, B, or C.) Temperature—25° C. or 40° C. Digestive periods—different.

26. Pepsin (A)—10 mgs. (0.1 c.c. of a 10% solution). Fibrin—B. Temperature— 25° C. Digestive period—6 hours. Neutralization—30 minutes after filtration was started. Compare with 23.

Hydrochloric	320	101	579	10	100	100	1	1	1
Chloric	603	40	357	19	40	62	3	3	3
Nitric	679	16	305	21	16	53	4	4	4
Sulfuric	852	4	144	27	4	25	5	5	5
Oxalic	543	46	411	17	46	71	2	2	2

27. Pepsin (A)—10 mgs. (0.1 c.c. of a 10% solution). Fibrin—C. Temperature—40° C. Digestive period—1 hour. Neutralization—30 minutes after filtration was started.

Hydrochloric	156	247	597	10	100	100	1	1	2
Chloric	305	191	504	20	77	84	3	2	3
Nitric	378	156	466	24	63	78	4	4	4
Sulfuric	573	70	357	37	28	60	5	5	5
Oxalic	224	171	605	14	69	101	2	3	1

28. Pepsin (D)—0.12 mg. (0.06 c.c. of a 0.2% solution). Fibrin—A. Room temperature. Digestive period—20 days (Aug. 18— Sept. 7, 1906). Neutralization—1 hour after filtration was started.

Hydrochloric	213	382	405	10	100	100	1	1	1
Chloric	358	290	352	17	76	87	3	3	3
Nitric	626	114	260	29	30	64	4	4	4
Sulfuric	766	10	224	36	3	55	5	5	5
Oxalic	256	356	388	12	93	96	2	2	2

Acid.	Weights of Products.			Relative Peptolysis.			Sequence.		
Nature.	R	N.P.	P.P.	R	N.P.	P.P.	R	N.P.	P.P.
				HCl product equals					
	mgs	mgs	mgs	10	100	100			

29. Duplication of the conditions of experiment 28, except that the amount of pepsin was **0.012 mg.** (0.06 c.c. of a 0.02% solution).

Hydrochloric	663	87	250	10	100	100	—	—	—
Chloric	897	25	78	14	29	31	—	—	—
Nitric [1]	—	—	—	—	—	—	—	—	—
Sulfuric	910	6	84	14	7	34	—	—	—
Oxalic	864	23	113	13	26	45	—	—	—

30. HCl controls for experiments 28 and 29. No pepsin.

Hydrochloric I	803	19	178	—	—	—	—	—	—
Hydrochloric II	809	24	167	—	—	—	—	—	—

31–34. Fourth series. **Pepsin—different preparations (B, C, or D); amounts—different. Fibrin—C; amounts—different.** Temperature—25° C. Digestive periods—different. Neutralization—30 minutes after filtration was started.

31. Fibrin—1 gram. Pepsin—B (0.05 c.c. of a concentrated glycerol extract of gastric mucosa). Digestive period—4 hours. Compare with 23.

Hydrochloric	91	330	579	10	100	100	1	1	1
Chloric	303	219	478	33	66	83	3	3	3
Nitric	486	151	363	53	46	63	4	4	4
Sulfuric	770	28	202	85	85	35	5	5	5
Oxalic	223	237	540	25	72	93	2	2	2

32. Fibrin—1 gram. Pepsin (C)—5 mgs. (0.05 c.c. of a 10% solution). Digestive period—1 hour. Compare with 24.

Hydrochloric	168	277	555	10	100	100	1	1	2
Chloric	410	131	459	24	47	83	3	3	3
Nitric	500	97	403	30	35	73	4	4	4
Sulfuric	763	16	221	45	6	40	5	5	5
Oxalic	272	153	575	16	55	104	2	2	1

33. Fibrin—1 gram. Pepsin (D)—50 mgs. (0.1 c.c. of a 50% solution). Digestive period—1 hour.

Hydrochloric	198	145	657	10	100	100	1	1	1
Chloric	411	85	504	21	59	77	3	3	3
Nitric	473	66	461	24	46	70	4	4	4
Sulfuric	686	21	293	35	14	45	5	5	5
Oxalic	254	121	625	13	83	95	2	2	2

[1] The results for nitric acid are not included in the table, because they appear to be incorrect, although no error was detected in obtaining them. The figures for weights of residue (R), neutralization precipitate (N.P.), and combined proteoses and peptones (P.P.) were respectively 601 mgs., 89 mgs., and 310 mgs.

Acid.	Weights of Products.			Relative Peptolysis.			Sequence.		
	R	N.P.	P.P.	R	N.P.	P.P.			
Nature				HCl product equals			R	N.P.	P.P.
	mgs	mgs	mgs	10	100	100			

34. Fibrin—2 grams. Pepsin (D)—50 mgs. (0.1 c.c. of a 50% solution). Digestive period—1 hour.

Hydrochloric	389	372	1239	10	100	100	1	1	2
Chloric	872	187	941	22	50	76	3	3	3
Nitric	1093	139	768	28	37	62	4	4	4
Sulfuric	1567	30	403	40	8	33	5	5	5
Oxalic	517	206	1277	13	55	103	2	2	1

35–39. Fifth series. Pepsin—different preparations (A, C, or D); amounts—different. Indicator—edestin or elastin. Temperature—25°C. Digestive periods—different

A. Indicator—edestin.

35. Pepsin (A)—10 mgs. (0.05 c.c. of a 20% solution). Digestive period—1 hour. Neutralization—30 minutes after filtration was started.

Hydrochloric	390	360	250	10	100	100	1	1	2
Chloric	447	341	212	11	95	85	3	2	3
Nitric	478	322	200	12	89	80	4	3	4
Sulfuric	684	181	135	18	50	54	5	5	5
Oxalic	395	255	350	10	71	140	2	4	1

B. Indicator—elastin

Acid	Weights of Products			Relative Peptolysis			Sequence.		
	R	P.P		R	P.P.		R	P.P.	
		Total	Precipitated		Total	Pr'cp'd		Total	Precipitated
Nature				HCl product equals					
	mgs	mgs	mgs	10	100	100			

36. Pepsin (C)—400 mgs. (dry). Digestive period—7 hours.

Hydrochloric	77	923	—	10	100	—	1	1	—
Chloric	104	896	—	14	97	—	3	3	—
Nitric	110	890	—	14	96	—	4	4	—
Sulfuric	90	910	—	12	99	—	2	2	—
Oxalic	121	879	—	16	95	—	5	5	—

37. Pepsin (A)—300 mgs. (dry). Digestive period—7 hours. Precipitation with alcohol—15 minutes after filtration was started.

Hydrochloric	354	646	416	10	100	100	1	1	3
Chloric	480	520	370	14	80	89	5	5	4
Nitric	469	531	350	13	82	84	4	4	5
Sulfuric	366	634	452	10	98	109	2	2	1
Oxalic	405	595	452	11	92	109	3	3	2

Acid.	*Weights of Products*			*Relative Peptolysis*			*Sequence.*		
		P.P			P.P.			P.P.	
Nature	R	Total	Precipitated	R	Total	Pr'cp'd	R	Total	Precipitated
				HCl product equals					
	mgs	mgs	mgs	10	100	100			

38. Pepsin (D)—300 mgs. (dry). Digestive period—7 hours. Precipitation with alcohol—15 minutes after filtration was started.

Hydrochloric	631	369	90	10	100	100	2	2	—
Chloric	699	301	100	11	82	111	4	4	—
Nitric	*730*	*270*	*140*	*12*	*73*	*156*	*5*	*5*	—
Sulfuric	620	380	—	10	103	—	1	1	—
Oxalic	660	340	—	11	92	—	3	3	—

39. Pepsin (A)—50 mgs. (0.2 c.c. of a 25% solution). Digestive period—10 hours.

Hydrochloric	821	179	—	10	100	—	1	1	—
Chloric	854	146	—	10	82	—	4	4	—
Nitric	864	136	—	11	76	—	5	5	—
Sulfuric	823	177	—	10	99	—	2	2	—
Oxalic	851	149	—	10	83	—	3	3	—

40–45. Second group of acid solutions—Hydrion (H˙) concentration equal to that of a 0.09% hydrochloric acid solution (at 25° C).

The names of the acids and data bearing on their concentrations are summarized below:

Acid	H˙: gram-atoms per 1000 liters	$\frac{\text{mol}}{\text{x}}$	%
Hydrochloric	25	39	0.09
Sulfuric	25	57	0.17
Phosphoric	25	60	0.16
Oxalic	25	29	0.31

Conditions of the experiments. All: Pepsin—D; amounts—different. Temperature—25° C.

40–42. First series. Fibrin—A or C; amounts—different[1]. Digestive periods—different.

[1] The fibrin was not perfectly dry, as was found after the series had been started, but contained 26 mgs. of water per gram of material. The 26 milligrams were accordingly apportioned among the products in order to make comparison with the remaining results more satisfactory.

Acid. Nature	Weights of Products. R mgs	N.P. mgs	P.P. mgs	Relative Peptolysis. R (HCl product equals 10)	N.P. (HCl product equals 100)	P.P. (HCl product equals 100)	Sequence. R	N.P.	P.P.

40. Pepsin—7.5 mgs. (0.075 c.c. of a 10% solution). Fibrin (C)—1 gram. Digestive period—5 hours. Neutralization—1 hour after filtration was started.

Hydrochloric	162	314	524	10	100	100	1	1	1
Sulfuric	657	105	238	41	33	45	4	4	4
Phosphoric	320	280	400	20	89	76	3	2	3
Oxalic	255	239	506	16	76	97	2	3	2

41. Repetition of the preceding experiment (40).

Hydrochloric	206	225	569	10	100	100	1	1	1
Sulfuric	704	61	235	34	27	41	4	4	4
Phosphoric	420	170	410	20	76	72	3	2	3
Oxalic	318	168	514	15	75	90	2	3	2

42. Fibrin (A)—2 grams. Pepsin—50 mgs. (0.1 c.c. of a 50% solution). Digestive period—1 hour. Neutralization—30 minutes after filtration was started. Compare with 34.

Hydrochloric	1706	57	237	10	100	100	1	1	1
Sulfuric	1954	11	35	11	19	15	4	4	4
Phosphoric	1878	25	97	11	44	41	3	2	3
Oxalic	1835	12	153	11	21	65	2	3	2

43–45. *Second series.* Elastin—1 gram. Pepsin—300 mgs. (dry). Digestive periods—7 hours. Precipitation with alcohol—15 minutes after filtration was started.

Acid. Nature	Weights of Products. R mgs	P.P. Total mgs	P.P. Precipitated mgs	Relative Peptolysis. R (HCl product equals 10)	P.P. Total (HCl product equals 100)	P.P. Prc'p'd (HCl product equals 100)	Sequence. R	P.P. Total	P.P. Precipitated
43.									
Hydrochloric	642	358	160	10	100	100	2	2	4
Sulfuric	639	361	410	10	101	256	1	1	2
Phosphoric	678	322	200	11	90	125	4	4	3
Oxalic	666	334	600	10	93	375	3	3	1

44. Repetition of the preceding experiment (43).

Hydrochloric	585	415	150	10	100	100	1	1	3
Sulfuric	619	381	350	11	92	233	2	3	1
Phosphoric	599	401	190	10	97	127	3	2	2
Oxalic	622	378	150	11	91	100	4	4	4

45. Repetition of experiments 43 and 44.

Hydrochloric	637	363	—	10	100	—	3	3	—
Sulfuric	629	371	—	10	102	—	2	2	—
Phosphoric	603	397	—	9	109	—	1	1	—
Oxalic	674	326	—	11	90	—	4	4	—

Swelling effects on fibrin in special experiments 28, 29, and 30. We have already commented upon the fact that the swelling of fibrin in our *isohydric* acid solutions was never uniform, either in rate or degree. In experiments 28, 29, and 30 special opportunity was afforded to observe *gradual* differences in bloating effects in equidissociated acid solutions. The digestive period for each experiment was 20 days. The pepsin amounted in each solution of one series (28) to 0.12 mg. and in each solution of the other (29) to only 0.012 mg. The swelling of the fibrin was decidedly unequal both in velocity and extent in each series of acid solutions. Appended is a summary indicating the order of swelling observed on the *third day:*

Experiments 28, 29, 30. Equidissociated acids of the first group, equal in $H^{\cdot}$ content to that of a 0.19% solution of hydrochloric acid. The sequence of expansive effects in the solutions of the three experiments is indicated below by the names of the acids with the corresponding figures for pepsin contents given in parenthesis, the greatest swelling having occurred in the first named solution, the least in the last named.

Third day. Hydrochloric (0.12), hydrochloric (0.012), *hydrochloric, containing no pepsin (2 controls, exp. 30)*, oxalic (0.12), oxalic (0.012), chloric (0.12), chloric (0.012), nitric (0.12), nitric (0.012). There was no obvious swelling in either of the sulfuric acid mixtures. A wide difference was observed between the bloating effects in the first hydrochloric (0.12) and the second nitric (0.012) acid mixtures.

The above sequence was also *the apparent order of digestion in the same acids* on the third day. The same sequence in each series of solutions was maintained to the end of the experiments. The sequence of swelling effects shows that the acids differed greatly in their bloating influences on fibrin. That pepsin favored the swelling is obvious, but that the enzyme helped it unequally and in the order of digestion in the acids is also evident.

All the residues continued to swell and increase perceptibly in bulk until about the 8th day. That the pepsin continued to affect the swelling perceptibly during this time was indicated by the fact that in all the solutions containing 0.12 mg. of the pepsin preparation the fibrin residue bloated more rapidly than

in the corresponding solution containing only 0.012 mg. The changes in the volumes of the residues, *i. e.*, both the swelling to a maximum and subsequent subsidence due to digestion were most rapid in the hydrochloric acid, and least rapid in the sulfuric acid, in the order given above for the acids. The residues in the solutions containing 0.012 mg. of the pepsin preparation were still swelling slowly after the residues in the solutions containing 0.12 mg. of it had previously swollen to their maximal bulks, and were decreasing in mass as a result of their digestion.

The intensity of coloration of the clear supernatant liquids varied with the differences in swelling. The solutions above the fibrin masses that were most swollen were, in general, a little more deeply colored than those above the fibrin masses that were least swollen. The chloric acid (0.12) solution was colored more deeply than any other, but the color was different in character from that of the others—a pinkish tinge having characterized it. The oxalic acid (0.12) solution was slightly cloudy; all the rest were clear.

The essential points in our observations in this connection are indicated above. We intend to repeat the experiments under various conditions and to discuss the significance of the results after more data have been accumulated. A definite relation between the swelling and the digestion of fibrin was indicated. See remarks on page 508.

Special digestive results of the experiments with the isohydrionic acid solutions. The results of the experiments in equipercentage, equimolecular, and equinormal acid solutions agreed in indicating that peptolysis depends largely upon the concentration of the hydrogen cation, but that it is also markedly affected by the associated anions or molecules, or both. An attempt was made to study these effects directly in the equidissociated acid solutions that were used in experiments 19-45. These experiments were performed under many different conditions, with the results summed up below.

Decided quantitative differences were observed in both groups of isohydrionic solutions, especially when the periods of digestion were short and the concentrations of pepsin relatively slight. The larger the active proportion of pepsin and the

longer the period of digestion of fibrin, or elastin, the nearer the peptolytic results approached quantitative equality. Wide quantitative differences were exhibited in two series of prolonged digestions through the agency of very slight proportions of pepsin. Different preparations of pepsin and different amounts of protein yielded quantitative peptolytic results of the same generally divergent character.

The nature of the protein was an important determinant of the rate, extent, and quality of peptolytic change in the isohydrionic acid solutions employed and under the given conditions of the experiments. The nearest approach to quantitative equality of peptolysis in the same solutions under various conditions was exhibited in the digestions of elastin.

The usual digestive sequence in the isohydrionic acid solutions was the following:

A. Five acids approximately equal in hydrion concentration to that of *0.2 per cent. HCl*:

a. With fibrin or edestin — Hydrochloric, oxalic, chloric, nitric, sulfuric.

b. With elastin—Hydrochloric and sulfuric; oxalic; chloric and nitric.[1]

The observed uniformity of these sequences in the many experiments performed is a very striking fact.

B. Four acids approximately equal in hydrion concentration to that of *0.1 per cent. HCl*:

a. Fibrin—Hydrochloric, oxalic, phosphoric, sulfuric.

[1] The quantitative results of the four experiments with elastin in the equidissociated acid solutions that were approximately equal in hydrion concentration to that of 0.2 per cent. HCl exhibited closer agreement than those obtained in the digestion of fibrin and edestin. This fact is shown below, where the figures for the total amounts of residues and dissolved portions of elastin for each acid in experiments 36–39 are summarized:

Acid.	Hydro-chloric.	Sulfuric.	Oxalic.	Chloric.	Nitric.
Residue (mgs.)...........	1883	1899	2037	2137	2137
Matter in solution (mgs.)..	2117	2101	1963	1863	1827

b. Elastin—Hydrochloric, phosphoric, sulfuric, oxalic.[1]

That peptolysis does not bear a simple relation to hydrion (H.) concentration, but that several factors influence it in every case, is very evident. The remarks made at the conclusion of the preceding section would be just as appropriate here.

General Results of Experiments 1–45 on Peptolysis.

Equipercentage data. The results of experiments 1–4 make it very evident that peptolysis is unequal both in rate and extent in a given group of acid solutions of equipercentage strength. In such solutions both rate and extent are different for different proteins. That these conclusions are correct for probably all percentage planes of concentration is suggested by the data for fibrin in the accompanying summary (page 526), which presents a few representative results of the many obtained in our experiments with isohydric acid solutions. Although the various solutions in a series were seldom exactly the same in their proportional contents of acid (as the figures in the second column show), their strength was so nearly identical in each instance that the slight percentage differences in concentration are devoid of practical import in this connection.

Equimolecular data. The results of experiments 5–8 are quantitatively as divergent as those of experiments 1–4. The accompanying summary (page 527), compiled from the data of the experiments with isohydric acid solutions, gives typical equimolecular results, which may be compared with the similar data of experiments 5–8. Such a comparison will show that the quantitative peptolytic differences in the $\frac{M}{30}$ solutions were not peculiar to their particular level of molecular concentration.

Equinormal (isohydric) data. The results of our experiments with acid solutions of equipercentage strength and of equimo-

[1] The figures for the total amounts of residues and dissolved portions of elastin for each acid in experiments 43–45 are summarized below (see the footnote on the opposite page.

Acid.	Hydrochloric.	Phosphoric.	Sulfuric.	Oxalic.
Residue (mgs.)	1864	1880	1887	1962
Matter in solution (mgs.)	1136	1120	1113	1038

SPECIAL SUMMARY OF COMPARATIVE DIGESTIVE RESULTS IN GROUPS OF ACID SOLUTIONS AT different planes OF PERCENTAGE EQUIVALENCE (experiments 9a-16).

Conditions of the experiments. Fibrin—A (except in experiment 16, in which fibrin B was used). Pepsin—A. Temperature—40° C. Digestive periods—4 hours (except in experiment 15, in which the period was 2 hours). Neutralization—1 hour after filtration was started.

No. of the Experiment.	Acid.				Weights of Products.			Pepsin.
	Nature.	Concentration.			R	N.P.	P.P.	
		%	mol / x	H· gram-atoms per 1000 liters	mgs.	mgs.	mgs.	mgs
14 a	Hydrochloric	0.09	40	24	71	248	681	1.6
	Phosphoric	0.08	120	16	127	243	630	1.6
13 a	Hydrochloric	0.09	40	24	49	219	732	4.0
	Phosphoric	0.08	120	16	82	213	705	4.0
13 a	Nitric	0.16	40	24	98	194	708	4.0
	Acetic	0.15	40	1	994	1	5	4.0
14 a	Nitric	0.16	40	24	169	238	593	1.6
	Acetic	0.15	40	1	984	2	14	1.6
11	Oxalic	0.23	40	17	69	69	862.	10.
	Arsenic	0.24	60	25	272	77	651	10.
	Sulfuric	0.25	40	34	258	57	685	10.
15	Oxalic	0.23	40	17	301	128	571	10.
	Arsenic	0.24	60	25	670	60	270	10.
	Sulfuric	0.25	40	34	628	64	308	10.
15	Nitric	0.32	20	48	212	136	652	10.
	Citric	0.32	60	3	954	1	45	10.
16	Oxalic	0.45	20	29	99	54	847	50.
	Arsenic	0.47	30	39	268	30	702	50.
	Sulfuric	0.49	20	61	507	8	485	50.
16	Acetic	0.60	10	1	951	2	47	50.
	Nitric	0.63	10	93	255	26	719	50.
	Citric	0.64	30	5	697	15	288	50.
9 a	Acetic	0.60	10	1	819	2	179	100.
	Nitric	0.63	10	93	74	62	864	100.
	Citric	0.64	30	5	347	9	644	100.
9 a	Chloric	0.84	10	93	58	81	861	100.
	Lactic	0.90	10	4	252	12	736	100.
16	Chloric	0.84	10	93	199	36	765	50.
	Lactic	0.90	10	4	650	14	336	50.

SPECIAL SUMMARY OF COMPARATIVE DIGESTIVE RESULTS IN GROUPS OF ACID SOLUTIONS AT different planes OF MOLECULAR EQUIVALENCE (experiments 9a-16.)

Conditions of the experiments. Stated on the opposite page.

No. of the Experiment.	Acid.				Weights of Products.			Pepsin.
	Nature.	Concentration.			R	N.P.	P.P.	
		mol/x	%	H· gram-atoms per 1000 liters	mgs	mgs.	mgs.	mgs.
12	Arsenic	120	0.12	16	830	8	162	5.
	Citric	120	0.16	2	967	0	33	5.
13 a	Sulfuric	80	0.12	19	509	83	408	4.
	Oxalic	80	0.11	9	628	39	333	4.
14 a	Sulfuric	80	0.12	19	644	84	272	1.6
	Oxalic	80	0.11	9	723	69	208	1.6
11	Phosphoric	60	0.16	25	42	56	902	10.
	Arsenic	60	0.24	25	272	77	651	10.
15	Phosphoric	60	0.16	25	92	180	728	10.
	Arsenic	60	0.24	25	670	60	270	10.
	Citric	60	0 32	3	954	1	45	10.
12	Hydrochloric	40	0.09	24	40	201	759	5.
	Hydriodic	40	0.32	25	148	103	749	5.
	Lactic	40	0.23	2	938	4	58	5.
13 a	Hydrochloric	40	0.09	24	49	219	732	4.
	Nitric	40	0.16	24	98	194	708	4.
	Acetic	40	0.15	1	994	1	5	4.
9 a	Phosphoric	30	0.33	40	40	36	924	100.
	Arsenic	30	0.47	39	97	25	878	100.
	Citric	30	0.64	5	347	9	644	100.
16	Phosphoric	30	0.33	40	49	43	908	50.
	Arsenic	30	0.47	39	268	30	702	50.
	Citric	30	0.64	5	697	15	288	50.
9 a	Sulfuric	20	0.49	61	122	55	823	100.
	Oxalic	20	0 45	29	47	32	921	100.
	Tartaric	20	0 75	6	204	14	782	100.
11	Hydrochloric	20	0.18	48	33	64	903	10.
	Hydriodic	20	0.64	49	57	93	850	10.
	Acetic	20	0.30	1	939	4	57	10.
9 a	Hydrochloric	10	0.36	94	35	40	925	100.
	Chloric	10	0.84	93	58	81	861	100.
	Nitric	10	0.63	93	74	62	864	100.
	Lactic	10	0.90	4	252	12	736	100.
	Acetic	10	0.60	1	819	2	179	100.
16	Hydrochloric	10	0.36	94	64	63	873	50.
	Chloric	10	0.84	93	199	36	765	50.
	Nitric	10	0.63	93	255	26	719	50.
	Lactic	10	0.90	4	650	14	336	50.
	Acetic	10	0.60	1	951	2	47	50.

lecular concentration make it evident that, all other conditions in a series being equal, identical *masses*, as well as equal *numbers of molecules*, of different acids fail to effect peptolysis to the same degree.

Peptolysis depends upon acidity. Acids that do not precipitate pepsin appear able to co-operate with the enzyme in zymolysis. All acids are molecularly alike in containing hydrogen. If peptolysis were dependent upon the *atoms* of hydrogen in the acids, irrespective of the *states* of the hydrogen atoms, peptic action would be quantitatively the same in equinormal (isohydric) solutions of different acids under identical conditions of digestion. That peptolysis is not determined, so far as the acid is concerned, by hydro*atomicity* alone, however, is indicated not only by the data of experiments 9a–18 but also by such divergent results in the foregoing summary, page 527, as those given in the last two series. The $\frac{N}{10}$ solutions of the five acids there referred to contain the same *amounts* of hydrogen, but the disparity in the peptolytic results could hardly be greater.

Equidissociated (isohydrionic) data. An examination of our quantitative data in any table makes it evident that, with all other conditions equal in a series, peptolysis was greatest, in general, in the solutions of the most dissociable acids employed and was least in the solutions of the acids that dissociate at best only slightly. That peptolysis is largely dependent upon hydrionic concentration was shown in all our experiments and is clearly indicated by the results in the following table of typical data from the summary on page 527, with accompanying figures for the degrees of dissociability.

Acid.					Weights of Peptolytic Products		
Nature.	Concentration.				R	N.P.	P.P.
	mol/x	%	$H^{\cdot}$ gram-atoms per 1000 liters.	Percentage of Dissociation.	mgs.	mgs.	mgs.
Hydrochloric	10	0.36	94	94.3	64	63	873
Chloric	10	0.84	93	93.3	199	36	765
Nitric	10	0.63	93	93.3	255	26	719
Lactic	10	0.90	4	3.7	650	14	336
Acetic	10	0.60	1	1.3	951	2	47

That peptolysis is quantitatively unequal, however, in any series of isohydrionic acid solutions under most conditions was shown by the results of experiments 19-45. Quantitative peptolytic differences were perhaps less marked in a series of isohydrionic acid solutions under given conditions than in a series of any other kind of solution of the same acids under identical conditions; but they were too wide under most conditions (except in the experiments with elastin) to be accounted for by unavoidable errors of manipulation and consequently were too great to permit the deduction that peptolysis bears a direct or obvious mathematical relation to hydrionic concentration. It seems evident that in peptolysis the helpful action of the hydrion of the acid is interfered with by harmful influences of the associated anions and (usually also) the molecules. The quantitative differences in each series of our peptolytic results in isohydrionic solutions may be explained in that way. If this deduction is correct, and we know of no reason to doubt its validity, the acid in which peptolysis can be accomplished with the greatest speed and to the greatest extent (under uniform optimum digestive conditions) must be that acid of highest dissociability whose anions (and molecules) interfere least with the co-operation of pepsin and hydrion in effecting protein hydrolysis.

The experiments already described make it evident that peptolysis is quantitatively unequal in any series of aqueous solutions of different acids, whether the acids are present in (1) *equal masses* or in *equal numbers* of (2) acid *molecules*, (3) hydrogen *atoms*, or (4) hydrogen *ions*. In order to test more directly the disturbing influence of associated anions and molecules we carried out the special experiments (46-53) summarized on pages 532–3.

Experiments with Solutions containing Two Acids in simple Molecular Proportions.

A. The results of the experiments (46–49) with solutions containing both *hydrochloric acid and sulfuric acid* (first series) warrant the following conclusions:

Under the conditions of these experiments the digestion of *fibrin* was much more rapid in $\frac{M}{20}$ hydrochloric acid than in $\frac{M}{20}$

sulfuric acid. The digestion of fibrin in $\frac{M}{20}$ hydrochloric acid was greatly interfered with by the presence of sulfuric acid, even when the concentration of the latter in the mixture was only $\frac{M}{100}$. The larger the proportion of sulfuric acid present in the digestive mixture, the greater the interference with peptolysis of the fibrin *in spite of the correspondingly increased H˙ concentration.*

Sulfuric acid solutions do not support the peptolysis of fibrin in the same degree that equivalent proportions of hydrochloric acid maintain it, but it is evident, also, that sulfuric acid positively interferes with the co-operation between hydrochloric acid and pepsin under the conditions of these experiments. The inhibitory effects of the sulfuric acid are seemingly due directly or indirectly to the qualities of the sulfuric acid anions or undissociated molecules, or both.

Decreasing quantities of neutralization precipitate were obtained from filtrates 2–6, in experiment 46 with fibrin, in spite of the favorable effects on the precipitation of acidalbumin that sulfates exercise.[1] Were it not for the latter fact, a considerable part of the observed decrease might be attributed to a more decided solvent action of the successively larger proportions of salts formed (2–6) in the neutralization process.

The digestion of *elastin* was practically the same in the $\frac{M}{20}$ solutions of sulfuric acid and hydrochloric acid. The differences were too slight for emphasis. This result accords with previous data to the effect that elastin may be digested practically as well in sulfuric acid as in hydrochloric acid, when solutions of equivalent proportions are compared.

With the result of previous experience in mind (p. 506) we supposed that the digestion of elastin would be improved by the addition of sulfuric acid to the hydrochloric acid solution and thought also that the digestion of the elastin in the mixture containing the maximal proportions, $\frac{M}{20}$, of both acids might be practically equivalent to that in an $\frac{M}{10}$ solution of either hydrochloric acid or sulfuric acid. The results did not bear out that expectation, however—rate and degree of digestion in a series were practically the same in each solution containing the two acids, in spite of the fact that the H˙ concentration was greater in each solution of the two acids than in either of the solutions containing only one

[1] Hawk and Gies, *loc. cit.*

of them. This outcome, like that with fibrin, implies the operation of conflicting influences in the solutions containing the two acids, however efficient each acid alone may be in sustaining peptolysis under its own auspices.

Our data on the digestion of elastin in this connection suggest that the digestive resultant of opposing influences, as exerted by both acids when together in solution, was approximately equal in each instance to the peptolytic result obtainable in the $\frac{M}{20}$ solution of either one. In the case of fibrin under the same circumstances the "digestive resultant" was less than the peptolytic result in the solution of hydrochloric acid alone, and greater than that in the pure solution of sulfuric acid. That the acid anions or molecules or both were deeply involved directly or indirectly in each instance seems evident.

B. That acid molecules are not *necessarily* inhibitory in peptolysis is strongly indicated by the results of the experiments (50–53) with solutions containing both hydrochloric acid and acetic acid, which may be summed up briefly as follows;

Under the conditions of these experiments the digestion of *fibrin* in $\frac{M}{20}$ hydrochloric acid was much more complete than in $\frac{M}{20}$ acetic acid. Acetic acid, even when present in the $\frac{M}{20}$ hydrochloric acid solution in a molecularly equivalent concentration, $\frac{M}{20}$, had no appreciably retarding influence or effect of any kind on the peptolysis of fibrin. The dissociation of the acetic acid in the mixtures employed was so slight in each case, that the proportion of acetic acid anions in each solution was inconsiderable. The $H^{\cdot}$ concentration was also practically the same, of course, in each mixture. The acetic acid in the solutions was almost entirely in *molecular* condition.

The figures for amounts of neutralization precipitates in experiment 50 might suggest that there was less production of acidalbumin from fibrin in the hydrochloric acid solutions containing the largest proportions of acetic acid than in those with the smallest. The rising proportions of acetate that were formed with the chlorid on neutralization of the filtrates (from 2 to 6) doubtless exerted sufficiently increased solvent action on the acidalbumin to account for the fairly uniform decrease (from 2 to 6) in the recovered amounts of neutralization precipitate.[1] (See remarks on page 501.)

[1] Hawk and Gies, *loc. cit.*

Experiments 46–53. With solutions containing two acids in simple molecular proportions. Conditions of the experiments. **All: Pepsin—D. Temperature—40° C. Volume of each solution—100 c.c.**

46–49. First series. Acids—hydrochloric and sulfuric.

A. With fibrin A.—Pepsin—10 mgs. (0.1 c.c. of a 10% solution). Digestive periods—3 hours. Neutralization—1 hour after filtration was started.

No. in Series.	Acid Solutions. Constituents. HCl $\frac{M}{10}$ c.c.	Second Acid $\frac{M}{10}$ c.c.	H_2O c.c.	Concentration. Total H˙ gram-atoms per 1000 liters.	Second Acid $\frac{mol}{x}$	Digestive Data. Weights of Products. R mgs.	N.P. mgs.	P.P. mgs.	Relative Peptolysis. $\frac{M}{10}$ HCl product equals R 10	N.P. 100	P.P. 100	Sequence. R	N.P.	P.P.
46. With fibrin.														
1	50	—	50	48	—	162	251	587	10	100	100	1	1	1
2	50	10	40	62	100	439	184	377	27	73	64	3	2	3
3	50	20	30	73	50	430	135	435	27	54	74	2	3	2
4	50	30	20	85	33	500	111	389	31	44	66	4	4	4
5	50	40	10	96	25	553	87	360	34	35	61	5	5	5
6	50	50	—	107	20	582	84	334	36	33	57	6	6	6
7	—	50	50	61	20	695	52	253	43	21	43	7	7	7
8	—	100	—	116	10	994	21	—	H_2SO_4 control; pepsin absent.					
9	100	—	—	94	—	960	11	29	HCl control; pepsin absent.					
47. Repetition of the preceding experiment (46).														
1	50	—	50	48	—	175		825	10		100	1		1
2	50	10	40	62	100	370		630	21		76	2		2
3	50	20	30	73	50	452		548	26		66	3		3
4	50	30	20	85	33	547		453	31		55	4		4
5	50	40	10	96	25	618		382	35		46	6		6
6	50	50	—	107	20	615		385	35		47	5		5
7	—	50	50	61	20	685		315	39		38	7		7
B. With elastin. 48. Pepsin—300 mgs. (dry). Digestive period—7 hours.														
1	50	—	50	48	—	451		549	10		100	5		5
2	50	10	40	62	100	419		581	9		106	2		2
3	50	20	30	73	50	431		569	10		104	3		3
4	50	30	20	85	33	442		558	10		102	4		4
5	50	40	10	96	25	460		540	10		98	7		7
6	50	50	—	107	20	453		547	10		100	6		6
7	—	50	50	61	20	403		597	9		109	1		1
8	—	100	—	116	10	985		15	H_2SO_4 control; pepsin absent.					
9	100	—	—	94	—	983		17	HCl control; pepsin absent.					
49. Repetition of the preceding experiment (48).														
1	50	—	50	48	—	391		609	10		100	2		2
2	50	10	40	62	100	419		581	11		95	6		6
3	50	20	30	73	50	401		599	10		98	3		3
4	50	30	20	85	33	407		593	10		97	4		4
5	50	40	10	96	25	434		566	11		93	7		7
6	50	50	—	107	20	408		592	10		97	5		5
7	—	50	50	61	20	353		647	9		106	1		1

50–53. Second series. Acids—hydrochloric and acetic.

A. With fibrin—A or C. Pepsin—10 mgs. (0.1 c.c. of a 10% solution). Digestive periods—3 hours. Neutralization—30 minutes after filtration was started.

No. in Series.	Acid Solutions. Constituents. HCl $\frac{M}{10}$ c.c.	Second Acid $\frac{M}{10}$ c.c.	H_2O c.c.	Concentration. Total H· gram-atoms per 1000 liters.	Second Acid $\frac{mol}{x}$	Digestive Data. Weights of Products. R mgs.	N.P. mgs.	P.P. mgs.	Relative Peptolysis ($\frac{M}{20}$ HCl product equals) R 10	N.P. 100	P.P. 100	Sequence. R	N.P.	P.P.
50.	**With fibrin A.**													
1	50	—	50	48	—	198	301	501	10	100	100	6	2	5
2	50	10	40	48	100	194	312	494	10	104	99	4	1	6
3	50	20	30	48	50	196	300[1]	504	10	100	101	5	3	4
4	50	30	20	48	33	193	297	510	10	99	102	3	4	3
5	50	40	10	48	25	187	262	551	9	87	110	1	5	2
6	50	50	—	48	20	191	222	587	10	74	117	2	6	1
7	—	50	50	0.9	20	945	12	43	48	4	9	7	7	7
8	—	100	—	1.3	10	961	45	—	CH_3COOH control; pepsin absent					
9	100	—	—	94	—	961	20	19	HCl control; pepsin absent.					
51.	**Repetition of the preceding experiment (50). With fibrin C.**													
1	50	—	50	48	—	87		913	10		100	6		6
2	50	10	40	48	100	74		926	9		101	5		5
3	50	20	30	48	50	45		955	5		105	2		2
4	50	30	20	48	33	48		952	6		104	3		3
5	50	40	10	48	25	50		950	6		104	4		4
6	50	50	—	48	20	44		956	5		105	1		1
7	—	50	50	0.9	20	958		42	110		5	7		7
B.	**With elastin. 52. Pepsin—300 mgs. (dry). Digestive period—7 hours.**													
1	50	—	50	48	—	474		526	10		100	6		6
2	50	10	40	48	100	407		593	9		113	1		1
3	50	20	30	48	50	418		582	9		111	4		4
4	50	30	20	48	33	432		568	9		108	5		5
5	50	40	10	48	25	417		583	9		111	3		3
6	50	50	—	48	20	414		586	9		111	2		2
7	—	50	50	0.9	20	570		430	12		82	7		7
8	—	100	—	1.3	10	982		18	CH_3COOH control; pepsin absent.					
9	100	—	—	94	—	983		17	HCl control; pepsin absent.					
53.	**Repetition of the preceding experiment (52).**													
1	50	—	50	48	—	415		585	10		100	1		1
2	50	10	40	48	100	428		572	10		98	4		4
3	50	20	30	48	50	435		565	10		97	5		5
4	50	30	20	48	33	427		573	10		98	3		3
5	50	40	10	48	25	441		559	11		96	6		6
6	50	50	—	48	20	420		580	10		99	2		2
7	—	50	50	0.9	20	603		397	15		68	7		7

[1] Our recorded result was 225, which was incorrect. In checking our figures it appeared that a written numeral 7 was mistaken for 1, and that the actual result obtained was that given above.

The digestion of *elastin* was better in $\frac{M}{20}$ hydrochloric acid than in $\frac{M}{20}$ acetic acid, but, as we found before, the difference between the two equivalent acids in peptolytic efficiency on elastin was not so great as it was in connection with fibrin. The acetic acid in the mixed solutions appeared to have little or no effect on the digestion of elastin, as in the case of fibrin. These observations will be extended.

Remarks on Peptolysis in Acetic Acid.

The results of these experiments with acetic acid throw new light on the observation frequently reported that peptolysis was almost negative in solutions of acetic acid. Such lack of peptolytic efficiency is apparently due chiefly to the low hydrion concentration of the acetic acid solutions employed. The acetic acid molecules and anions, in the proportions taken in these experiments, are practically inert. It is obvious that peptolysis of fibrin and elastin is neither materially favored nor interfered with by moderate amounts of acetic acid, a fact which indicates that the purely *chemical* phases of the normal gastric digestive process are practically unaffected by vinegar in the quantities ordinarily ingested. *Secretory* conditions, however, are modified no doubt. We expect shortly to report from this laboratory some results in this particular connection.

All our previous digestive experiments with pure solutions of acetic acid have shown that peptolysis of *fibrin* proceeded very slowly in each of the acetic acid solutions employed and that the process for *elastin* in acetic acid solutions of the same concentrations was also relatively slow, but less so than for fibrin. We have just observed that acetic acid was practically inert in the peptolysis of fibrin or elastin in the hydrochloric-acetic solutions of experiments 50-53.

That it is impossible in such work to draw significant conclusions without basing them on the results of numerous experiments is shown by the appended data of an experiment with fibrin in which increasing proportions of a pepsin preparation were used in equal volumes of $\frac{M}{10}$ solution of acetic acid under uniform digestive conditions.

54 *Conditions of the experiment.* Fibrin—A. Pepsin—D. Temperature—40° C. Digestive period—3 hours. Neutralization—30 minutes after filtration was started.

Digestive Mixtures.			Weights of Digestive Products.		
No.	$\frac{N}{16}$ CH_3COOH	Pepsin—D[1]	R	N.P.	P.P.
	c.c.	mgs.	mgs.	mgs.	mgs.
1	100	None	961	45	—
2	100	500	677	41	282
3	100	1000	558	56	386
4	100	2000	390	105	505

The results of this experiment (54) show very clearly that even acetic acid will help actively in peptolysis if sufficient pepsin is present in the solution with it. The significance of this and related observations will be discussed with additional data in a future paper.

Effects of the Peptolytic Products of one Protein on the Peptic Digestion of Another.

That all of the quantitative results of digestion are automatically influenced by the products of the process is well understood. That such modifications have occurred unequally in every series of our experiments is certain. Before proceeding to a consideration of our tryptolytic data, we desire to record here the results of two preliminary experiments on the influence of the peptolytic products of one protein on the peptic digestion of another, a study we have planned to extend. In the experiments referred to below our proteins were fibrin and elastin; one of which is readily digested by small proportions of pepsin, whereas the other is much more resistant to peptolysis. We selected this pair of proteins in order to make the digestive conditions of our experiments as unfavorable as possible to the outcome we expected. We assumed that, in general, the more digestible the proteins might be, the more they would interfere with each other's peptolysis.

Until we are prepared to present further data in this and related connections, it will be sufficient here to call attention to the fact that in experiments 55–56 there was appreciable interference with the digestion of one protein or the other, or both, in every solution containing portions of each protein.

[1] It should not be forgotten in this particular connection that our pepsin preparation was composed chiefly of non-pepsinic material. The actual quantity of pepsin in the largest amount of the preparation taken was very slight therefore. Its proportion in the digestive mixtures was probably no greater than that frequently present in normal gastric juice.

Experiments 55–56. Effects of the peptolytic products of one protein on the peptic digestion of another. Conditions of the experiments. All: Hydrochloric acid, 0.2 %—100 c.c. Pepsin—D; 50 mgs. (0.2 c.c. of a 25% solution). Temperature—40°C. Digestive periods—1 hour and 45 minutes. Protein indicator—fibrin (B), elastin, or both; amounts—different.

No. in the Series	Protein taken.			Protein recovered.		Calculated amounts of residue.			
	Fibrin	Elastin	Total	Weight	%	Approximate quantity of residue yielded by the proteins in the absence of each other[1]		Total	Excess of total residue obtained over that calculated
						Fibrin	Elastin		
	gram	gram	gram	gram		gram	gram	gram	gram
					55.				
1	—	0.25	0.25	0.193	77.20	—	—	—	—
2	2.00	—	2.00	0.717	35.85	—	—	—	—
3	1.75	0.25	2.00	1.196	59.80	0.627	0.242	0.869	0.327
4	1.50	0.50	2.00	1.544	77.20	0.538	0.483	1.021	0.523
5	1.25	0.75	2.00	1.736	86.80	0.448	0.724	1.172	0.564
6	1.00	1.00	2.00	1.812	90.60	0.358	0.966	1.324	0.488
7	0.75	1.25	2.00	1.857	92.85	0.269	1.207	1.476	0.381
8	0.50	1.50	2.00	1.872	93.60	0.179	1.449	1.628	0.244
9	0.25	1.75	2.00	1.902	95.10	0.090	1.690	1.780	0.122
10	—	2.00	2.00	1.932	96.60	—	—	—	—
11	0.25	—	0.25	0.056	22.40	—	—	—	—
					56.				
1	2.00	—	2.00	0.723	36.15	—	—	—	—
2	1.00	—	1.00	0.376	37.60	—	—	—	—
3	0.75	—	0.75	0.260	34.66	—	—	—	—
4	0.50	—	0.50	0.188	37.60	—	—	—	—
5	0.25	—	0.25	0.087	34.80	—	—	—	—
6	—	2.00	2.00	1.929	96.45	—	—	—	—
7	—	1.00	1.00	0.932	93.20	—	—	—	—
8	—	0.75	0.75	0.689	91.87	—	—	—	—
9	—	0.50	0.50	0.422	84.40	—	—	—	—
10	—	0.25	0.25	0.193	77.20	—	—	—	—
11	0.25	1.75	2.00	1.905	95.25	0.090	1.687	1.777	0.128
12	1.00	1.00	2.00	1.747	87.35	0.362	0.964	1.326	0.421
13	1.75	0.25	2.00	1.084	54.20	0.633	0.241	0.874	0.210

II—Comparative Tryptolysis.

Groups of Basic Solutions Employed.

The results of our peptic experiments were so much alike in essential physico-chemical features in all the types of acid solutions employed that, in turning to tryptolysis, we directed our attention at once to the activity of trypsin in equidissociated (isohydroxidionic) solutions of bases. This type of basic solution is obviously the most important for work of this kind and, judging from the results of our experiments with the acids, its use promised to give equally significant data.

[1] Calculated from the corresponding data for the digestion of *two grams* of each protein. The calculated amounts of residue on this basis are the *maximal* theoretical quantities.

Type of Basic Solutions Employed.

Four groups of basic solutions were used, in each of which the hydroxidion (OH′) concentration of the solutions was equal to that of a solution of sodium carbonate of definite percentage strength, as follows: Group A—0.5 per cent; Solution B—0.25 per cent; Group C—1 per cent; Group D—0.25 per cent; Group E—1 per cent. Our proteolytic results in these solutions were not materially affected by the slight solvent action of the bases alone.

The names of the bases of each group, with data bearing on their concentrations and their solvent action on fibrin[1] and elastin, are summarized below:

Base.	Concentration.			Residues after Treatment With the Solutions in the Absence of Trypsin	
Groups.	OH′ gram-mols per 1000 liters	mol / x	%	Bases–100 c.c. Fibrin (C)—1 gram Temperature–25° C. Period of exposure to the bases 24 hours	Bases–100 c.c. Elastin–1 gram. Temperature—25° C. Period of exposure to the bases—7 hours
Group A:				mgs.	mgs.
Sodium carbonate	4.65	21	0.50	984	1003
Ammonium hydroxid	4.65	1	3.29	978	1007
Potassium hydroxid	4.65	215	0.026	989	995
Tetra-ethyl ammonium hydroxid	4.65	200	0.07	991	1010
Conin	4.65	48	0.27	996	1003
Piperidin	4 65	55	0.15	981	1006
Piperazin	4.65	3	2.93	985	1015
Ethylene di-amin	4.65	4	1.55	973	1003
Tri-methyl amin	4 65	3	1.74	953	994
Solution B:					
Sodium carbonate	3 38	42	0 25	984	1006
Group C					
Ammonium hydroxid	5 96	0 7	5.40	973	996
Potassium hydroxid	5 96	168	0.033	984	996
Group D:					
Sodium carbonate	3 38	42	0.25	—	—
Ammonium hydroxid	3 38	2	1.75	—	—
Potassium hydroxid	3 38	296	0.019	—	—
Conin	3 38	83	0.15	—	—
Group E:					
Sodium carbonate	5 96	11	1.00	—	—
Ammonium hydroxid	5.96	0.7	5.40	—	—
Potassium hydroxid	5.96	168	0.033	—	—
Conin	5.96	30	0.42	—	—

[1] The neutralization precipitates obtained in the filtrates from the fibrin in the usual way were too slight for gravimetric determination.

EXPERIMENTS 57-66.[1] WITH EQUIDISSOCIATED (ISOHYDROXIDIONIC) BASIC SOLUTIONS.

Conditions of the experiments. **All: Temperature—25° C.**

57–61. *First series.* With fibrin—B or C. Trypsin—A, B, or C. Digestive periods—2 hrs., 45 minutes. Neutralization—1 hour after filtration was started.

Base.	Weights of Products.			Relative Tryptolysis.			Sequence.		
	R	N.P.	P.P.	R	N.P.	P.P.	R	N.P.	P. P.
	mgs.	mgs.	mgs.	Na_2CO_3 product equals 10	100	100			
57. Fibrin—C. Trypsin (A)—450 mgs. (dry).									
Group A:									
Sodium carbonate	55	206	739	10	100	100	3	3	4
Ammonium hydroxid	66	151	783	12	73	106	4	4	3
Potassium hydroxid	352	135	513	64	66	69	9	6	9
Tetra-ethyl ammonium hydroxid	329	136	535	60	66	72	8	5	8
Conin	84	255	661	15	124	89	5	1	7
Piperidin	101	213	686	18	103	93	6	2	6
Piperazin	30	120	850	5	58	115	2	8	1
Ethylene di-amin	29	125	846	5	61	114	1	7	2
Tri-methyl amin	162	115	723	29	56	98	7	9	5
Solution B:									
Sodium carbonate	92	189	719	—	—	—	—	—	—
Group C:									
Ammonium hydroxid	88	108	804	—	—	—	1	2	1
Potassium hydroxid	317	136	547	—	—	—	2	1	2
58. Fibrin—C. Trypsin (A)—300 mgs. (dry).									
Group A:									
Sodium carbonate	132	230	638	10	100	100	4	3	3
Ammonium hydroxid	106	190	704	8	83	110	2	9	2
Potassium hydroxid	445	142	413	34	62	65	9	8	9
Tetra-ethyl ammonium hydroxid	422	142	436	32	62	68	8	7	8
Conin	183	228	589	14	99	92	6	4	5
Piperidin	177	243	580	13	106	91	5	2	6
Piperazin	55	220	725	4	96	114	1	5	1
Ethylene di-amin	120	263	617	9	114	97	3	1	4
Tri-methyl amin	233	200	567	18	87	89	7	6	7
Solution B:									
Sodium carbonate	172	248	580	—	—	—	—	—	—
Group C:									
Ammonium hydroxid	175	177	648	—	—	—	1	1	1
Potassium hydroxid	406	151	443	—	—	—	2	2	2

[1] In some of these experiments the protein was not perfectly dry when it was used. The proportion of moisture was ultimately determined carefully in such instances, however, and the few milligrams of moisture per gram of fibrin were apportioned among the products to make the total originally intended. In no case was the amount of such moisture more than 26 milligrams per gram of material. See footnote, page 520.

The trypsin preparations contained material that was precipitated in the neutralization process. Due correction was made in this connection, therefore, after individual determinations of the amount of such contribution to the neutralization precipitate.

Base.	Weights of Products. R	N.P.	P.P.	Relative Tryptolysis. R	N.P.	P.P	Sequence. R	N.P.	P.P.
				Na$_2$CO$_3$ product equals					
	mgs.	mgs.	mgs.	10	100	100			
59. Fibrin—C. Trypsin (B)—300 mgs. (dry).									
Group D:									
Sodium carbonate	35	86	879	10	100	100	2	2	1
Ammonium hydroxid	23	346	631	7	402	72	1	1	4
Potassium hydroxid	237	38	725	68	44	82	4	4	3
Conin	50	73	877	14	85	100	3	3	2
Group E:									
Sodium carbonate	28	107	865	10	100	100	2	2	2
Ammonium hydroxid	28	611	361	10	571	42	1	1	4
Potassium hydroxid	86	89	825	31	83	95	4	3	3
Conin	34	58	908	12	54	105	3	4	1
60. Repetition of experiment 59. Fibrin—B.									
Group D:									
Sodium carbonate	181	11	808	10	100	100	1	2	1
Ammonium hydroxid	209	—	791	12	—	98	2	4	2
Potassium hydroxid	422	9	569	23	82	70	4	3	4
Conin	312	15	673	17	136	83	3	1	3
Group E:									
Sodium carbonate	403	14	583	10	100	100	3	2	3
Ammonium hydroxid	598	10	392	15	71	67	4	3	4
Potassium hydroxid	314	15	671	8	107	115	2	1	2
Conin	233	6	761	6	43	131	1	4	1
61. Repetition of experiments 59 and 60. Trypsin—C. Fibrin—B									
Group D:									
Sodium carbonate	136	16	848	10	100	100	1	1	2
Ammonium hydroxid	139	2	859	10	12	101	2	3	1
Potassium hydroxid	305	—	695	22	—	82	4	4	4
Conin	203	15	782	15	94	92	3	2	3
Group E:									
Sodium carbonate	335	18	647	10	100	100	3	1	3
Ammonium hydroxid	601	8	391	18	44	60	4	3	4
Potassium Hydroxid	231	7	762	7	39	118	2	4	2
Conin	124	13	863	4	72	133	1	2	1

62-66. *Second series.* With elastin. Trypsin—A or C. Digestive periods—different.

Base.	Weights of Products. R	P.P.	Relative Tryptolysis. R	P.P.	Sequence. R	P.P
			Na$_2$CO$_3$ product equals			
	mgs.	mgs.	10	100		
62. Trypsin (A)—300 mgs. (dry). Digestive period—3 hours, 45 minutes.						
Group A:						
Sodium carbonate	136	864	10	100	6	6
Ammonium hydroxid	794	206	58	24	8	8
Potassium hydroxid	135	865	10	100	4	4
Tetra-ethyl ammonium hydroxid	135	865	10	100	5	5
Conin	98	902	7	104	3	3
Piperidin	96	904	7	105	2	2
Piperazin	72	928	5	107	1	1
Ethylene di-amin	784	216	56	25	7	7
Tri-methyl amin	1003	—	74	—	9	—

Base.	Weights of Products.		Relative Tryptolysis.		Sequence.	
	R	P.P.	R	P.P.	R	P.P.
			Na_2CO_3 product equals			
	mgs.	mgs.	10	100		
62 (*Continued*)						
Solution B:						
Sodium carbonate	89	911	—	—	—	—
Group C:						
Ammonium hydroxid	970	30	—	—	2	2
Potassium hydroxid	147	853	—	—	1	1

63. Trypsin (A)—200 mgs. (dry). Digestive period—7 hours.

Group A:						
Sodium carbonate	91	909	10	100	6	6
Ammonium hydroxid	763	237	84	26	8	8
Potassium hydroxid	64	936	7	103	4	4
Tetra-ethyl ammonium hydroxid	65	935	7	103	5	5
Conin	49	951	5	105	3	3
Piperidin	41	959	5	106	1	1
Piperazin	47	953	5	105	2	2
Ethylene di-amin	749	251	82	28	7	7
Tri-methyl amin	1003	—	110	—	9	—
Solution B:						
Sodium carbonate	39	961	—	—	—	—
Group C:						
Ammonium hydroxid	951	49	—	—	2	2
Potassium hydroxid	53	947	—	—	1	1

64. Trypsin (C)—200 mgs. (dry). Digestive period—2 hours, 35 minutes.

Group D:						
Sodium carbonate	64	936	10	100	1	1
Ammonium hydroxid	81	919	13	98	3	3
Potassium hydroxid	103	897	16	96	4	4
Conin	80	920	12	98	2	2
Group E:						
Sodium carbonate	682	318	10	100	3	3
Ammonium hydroxid	944	56	14	18	4	4
Potassium hydroxid	66	934	1	294	1	1
Conin	76	924	1	290	2	2

65. Trypsin (A)—200 mgs. (dry). Digestive period—6 hours. Compare with 64.

Group D:						
Sodium carbonate	87	913	10	100	1	1
Ammonium hydroxid	165	835	19	91	3	3
Potassium hydroxid	176	824	20	90	4	4
Conin	111	889	13	97	2	2
Group E:						
Sodium carbonate	949	51	10	100	2	2
Ammonium hydroxid	983	17	10	33	3	3
Potassium hydroxid	93	907	1	1780	1	1
Conin	985	15	10	29	4	4

66. Trypsin (A)—195 mgs. (dry). Digestive period—6 hours. Compare with 65.

Group E:						
Sodium carbonate	841	159	10	100	2	2
Ammonium hydroxid	972	28	12	18	4	4
Potassium hydroxid	97	903	1	568	1	1
Conin	949	51	11	32	3	3

On the swelling effects of the basic solutions on fibrin and elastin. In all the experiments in which *fibrin* was used the protein gradually increased in bulk in each solution. The bloating effects *differed* greatly in degree, however.

Elastin was swollen by some of the bases, but not by others. In experiment 63, for example, swelling occurred unequally in the solutions (Group A) of sodium carbonate, ammonium hydroxid, piperazin, ethylene di-amin and tri-methyl amin, but did not appear in the solutions (Group A) of potassium hydroxid, tetra-ethyl ammonium hydroxid, conin, and piperidin. Swelling occurred in ammonium hydroxid (Group C), but not in sodium carbonate (Solution B) or potassium hydroxid (Group C). Elastin did not swell in any of these solutions when trypsin was absent. In general, digestion appeared to be most rapid and complete in the solutions which had no bloating effect on the elastin. There were notable exceptions, however.

It will be observed that the solutions in which elastin was swollen contained bases in relatively high molecular concentrations. On the other hand, swelling failed to occur in the solutions containing relatively small proportions of strong bases, and consequently, of small proportions of *undissociated* basic molecules. These facts suggest that the swelling of elastin was influenced not only by the trypsin but also by the undissociated base in each solution.

Digestive results of the experiments with the equidissociated (isohydroxidionic) basic solutions. Wide quantitative differences were noted in the tryptic digestions. In some respects there was more uniformity in the experiments with elastin than in those with fibrin, although the longer periods of exposure to tryptolytic influences in the former series may account for the fact stated.

The approximate order of tryptolytic efficiency of the basic solutions was the following:

A. With fibrin—*Piperazin*,[1] ethylene di-amin, ammonium hydroxid, and sodium carbonate; conin and piperidin; tri-

[1] Bredig (*loc. cit.*, p. 307) found that piperazin (di-ethylene di-amin) in aqueous solution dissociates like a mon-acid base, *i. e.*, like NaOH, for example. One molecule of piperazin dissociates, then, into one OH′ and an electropositive ion containing an *undissociated* hydroxyl group. The superior digestive results obtained in our piperazin solutions suggest that perhaps the latter hydroxyl group was dissociated from one cation

methyl amin; tetra-ethyl ammonium hydroxid and potassium hydroxid.

B. With **elastin**—*Piperazin*, piperidin, conin, potassium hydroxid, tetra-ethyl ammonium hydroxid and sodium carbonate; ethylene di-amin and ammonium hydroxid; tri-methyl amin.

Rate, extent, and quality of tryptolysis were influenced greatly by the nature of the protein. Elastin was in general less readily digested than fibrin. The digestions of fibrin and elastin were relatively about the same in the solutions of piperazin, sodium carbonate and conin. Digestion of fibrin was very poor in tri-methyl amin solution, but elastin did not digest in it at all. Fibrin was digested much more readily than elastin in the solutions of ethylene di-amin and ammonium hydroxid, in which the tryptolysis of elastin was particularly poor. On the other hand tryptolysis of elastin was relatively much better than that of fibrin in the solutions of piperidin, tetra-ethyl ammonium hydroxid, and potassium hydroxid. These differences were noted in the use of the same preparation of trypsin (A).

Bases of the same general nature yielded practically identical digestive results in a series. An exception was the marked divergence in this respect between piperazin and ethylene di-amin in the digestion of elastin.

That tryptolysis does not bear a simple relation to hydroxidion (OH′) concentration, but that it is markedly influenced by associated factors, is evident. The conditioning factors in our tryptolytic experiments were probably analogous to those of our peptic digestions.

General Considerations.

All the experiments described in this paper were carried out with the greatest possible regard for quantitative accuracy in every detail. The quantitative disparities observed among the results of each experiment are believed to express real proteolytic differences under the prevailing conditions and not incidental differences due to faulty technic. The uniformity of

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after another (and thus rendered zymolytically efficient) during the digestive process, as fast as hydroxylions were removed from ionic condition by combination with digestive products. The same may be said of ethylene di-amin, although the meager digestion of elastin in its solutions indicates the operation of influences not considered above.

the differences noted for each acid or base among the results of the several groups of experiments with a particular protein, when the differences are converted into common terms, enforces the validity of this deduction.

We have endeavored to explain definitely the quantitative proteolytic differences observed, but cannot satisfactorily ascribe them either to a single influence or to any particular set of influences. Thus, peptolysis in a series of acid solutions does not show a simple relation to any degree of concentration, whether the acid solutions are equivalent in percentage or molecular strengths, whether they exhibit identical proportions of acidity or conduct an electric current to the same degree. This statement is also true of tryptolysis. Our digestive results likewise bear no obvious relation to the heat of ionization or to the heat of formation of the acids or bases, nor to such other physico-chemical properties as the solution tension, velocity, or conductivity of the cations or anions. That some or all of these factors are operative is probable. That the hydrogen cation supports peptolysis, that the hydroxyl anion may stimulate tryptolysis, is evident. That the associated anions or molecules or both usually inhibit peptolysis, that the accompanying cations or molecules or both retard tryptolysis, seems equally probable. It is possible that the use of solutions of greater dilution than those employed in our work heretofore might yield results which would clearly reveal the leading influences involved in digestive proteolysis.

Our results show that the chemical operations in peptolysis and tryptolysis are very complicated, and that the quantitative outcome in each case is the resultant of many forces. Our experiments have proved conclusively that the nature of a protein is one of the important factors in its zymolysis. It is possible that the dissimilar digestive results for the proteins employed were due chiefly to different degrees of affinity between the proteins and their zymolytic products on one side and the acids or bases in each group on the other. Such differences of chemical combination might result in variable degrees of susceptibility of a protein and its zymolytic products to the active enzyme in any series of acid or basic solutions. See experiments 55-56.

The fact that each of our protein products differed widely from the others in the velocity, quality, and extent of its digestion in a given series of equivalent acid or basic solutions makes it clear that no important generalizations can yet be offered to explain the behavior of the acids in peptolysis or the bases in tryptolysis. Our results indicate strongly that numerous current opinions relating to digestive proteolysis that have been derived from the data of investigations in each of which a single protein was used, as, for example, such as were intended to show the effects of various substances on the purely chemical phases of normal gastric digestion, need revision.

We intend to continue this particular phase of our study with an investigation of the comparative zymolytic behavior of *many different proteins* in samples of the same groups of acid and also basic solutions that will be made in large volumes, in the hope of finding the important common factors in proteolysis and also of discovering the relationships of the significant influences that may be peculiar to the various proteins and to the acids or bases employed. The effects of the various acid and basic solutions on enzymes will also be considered.

Because of the purposes just indicated we have refrained from suggesting more than one or two of our own present opinions in these connections. The bare statement of our methods and results has required so much space that we shall also postpone reference to the related work of other observers until the data of our further study of this matter warrants renewed discussion of the subject.

Summary of Main Conclusions.

Peptolysis of either fibrin, edestin or elastin is quantitatively unequal in a series of aqueous solutions of different acids under any uniform digestive conditions. Striking disparities in the velocity, quality, and extent of digestion of these proteins occur in solutions of common acids whether the acids are present in the solutions in equal *masses* (equipercentage), or in equal *numbers* of acid *molecules* (equimolecular), hydrogen *atoms* (equinormal), or hydrogen *ions* (equidissociated).

Tryptolysis of fibrin and elastin is markedly unequal in equivalent solutions of bases.

Among the conditioning influences that were obviously influential in all our zymolytic experiments were the character, state, and strength of the acid or base, the quality and concentration of the enzyme, the duration of the period of digestion, the temperature of the digestive mixture and the nature of the protein. No doubt the different digestive products themselves exerted unequal influences as the proteolytic transformation proceeded.

The hydrogen ion is the favorable acid factor in peptolysis. The associated anions or molecules (or both) appear to interfere (as a rule) with the peptic process, and their divergent influences seem to account, in part at least, for the quantitative disparities noted in each digestive series of these experiments. It has been shown, however, that *acetic* acid *molecules* are practically without influence, under certain conditions, on the peptolysis of fibrin or elastin in solutions of hydrochloric acid—a suggestion that the purely *chemical* phases of the normal gastric digestive process are practically unaffected by vinegar in the quantities ordinarily ingested. The effects of acetic acid and of vinegar on the *secretory* process in the stomach will be investigated.

In tryptolysis the hydroxyl anion is the favorable basic factor, and the associated cations or molecules (or both) seem to exercise deterrent influences.

In general harmony with the observed digestive disparities, there were marked inequalities of the swelling effects on fibrin in every equivalent series of acid or basic solutions. Bloating influences on fibrin were due primarily to the acid or base, but were more pronounced in the presence of enzyme. Elastin did not swell perceptibly in either the acid or basic solutions employed, but did so in the latter when trypsin was present.

The parts played by the various ions and molecules in peptolysis, or tryptolysis, or in the swelling of the proteins, have not been considered experimentally in any special way. Whether the ions or molecules or both affect the proteins, the enzymes, the water, or all collectively, in the swelling and hydrolytic processes will be considered at a more favorable opportunity.

In a given series of equivalent acid or basic solutions under uniform digestive conditions the degree and sequence of zymolysis of fibrin were strikingly different from those of the digestion of

elastin. This fact necessitates thorough study of the zymolysis of many proteins in samples of the same equivalent acid and basic solutions, before general deductions relating to peptolysis, or tryptolysis, or to influences on them, are permissible. Such a study has been inaugurated.

ON THE COMPOSITION OF TOXICODENDROL.[1]

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(Received for publication, December 14, 1906.)

Up to the present time only three important chemical investigations of the poison ivy plant have been recorded, namely, those of Khittel,[2] Maisch,[3] and Pfaff.[4] The object of their work was to obtain the poisonous principle of the plant, and their results may be briefly stated as follows: Khittel thought that the poison was a volatile alkaloid, but he did not prove by physiological evidence that he had obtained a poisonous substance. Maisch obtained a volatile acid, which he called *toxicodendric acid*, by distilling a water extract of the plant. His acid was similar to formic and acetic acids, but more like the latter. He produced physiological evidence of a doubtful character to show that it was poisonous. Pfaff showed that the volatile acid of Maisch was probably impure acetic acid containing traces of the poisonous substance which he thought was not volatile. He got from the plant a non-volatile oil, *toxicodendrol*, which he showed by most reliable tests upon himself to be, or to contain, the poison.

The investigation of the writers was undertaken to see if more light could be thrown on the chemical nature of toxicodendrol. Soon after commencing work, however, it became apparent that the poison could be more intelligently studied if the substances associated with it in the plant were first identified.

[1] We are indebted to the Carnegie Institution of Washington for aid in this work. This paper is an abstract of a dissertation submitted to the Board of University Studies of the Johns Hopkins University by W. A. Syme for the degree of Doctor of Philosophy. For a more detailed account of the work reference is made to the dissertation.

[2] *Amer. Journ. of Pharm.*, p. 542, 1858.

[3] *Proc. Amer. Pharm. Assn.*, p. 166, 1865; *Amer. Journ. of Pharm.*, p. 4, 1866.

[4] *Journ. of Exper. Med.*, ii, p. 181, 1897.

The scope of the work was therefore extended to an investigation of the other constituents of the plant.

The crude material for this work was prepared by Messrs. Parke, Davis & Co., of Detroit, Michigan, according to special instructions submitted to them: 67.5 pounds of fresh leaves and flowers of poison ivy were collected near Detroit and carefully inspected by a competent botanist. This material was thoroughly macerated and put into ten-liter bottles with ether. The mass was thoroughly shaken, water being added to make it more mobile. The ether was then separated, and the extraction was repeated three times in the same way to insure complete removal of the toxicodendrol. The ether extracts were combined, thoroughly dried with anhydrous sodium sulphate, and the ether was distilled off, the temperature being kept below 40° C. during the entire distillation. The residue after the removal of the ether was a thick, black, tar-like mass, weighing 3 pounds, 11 ounces. In extracting the plant, about 24 gallons of ether were used. It is a significant fact in regard to the volatility of the poison that during the process of preparing this material none of the employees engaged in the work were in any way affected, since proper precautions were taken and the utensils were handled with rubber gloves.

The crude ether extract, which will be designated as the "original material," was shipped to Baltimore in August and was kept in a cool place until November, when the investigation was begun. When the bottle was opened by Dr. Syme there seemed to be an escape of a vapor, and a nauseating odor suggesting crushed green leaves pervaded the atmosphere. Some days later irregular red patches appeared on his face though a mask of cotton cloth was worn during the work, and his hands were protected by rubber gloves.

The first experiment was to try to distill the toxicodendrol from the original material with the aid of a vacuum pump. At a temperature of 140°–150° C. and a pressure of 2 millimeters, the material carbonized and nothing distilled over.

It was then thought that perhaps the oil could be converted into an ester which might be more volatile and could be distilled out. Twenty grams of the original material were dissolved in 100 c.c. of absolute alcohol containing 3 grams of hydrochloric

acid gas, and the mixture was heated 10 hours on a water-bath under a return condenser. After the heating, the mixture had a delightful ethereal odor. The flask was corked and left standing several weeks while other work was in progress. The ester solution was then put into a vacuum desiccator over sulphuric acid and the alcohol evaporated. A black, tarry, solid mass was left having the ester odor. It was extracted with warm water and filtered from insoluble tar. The filtrate had a green color and the ethereal odor. It was shaken out with ether; the ether layer had a blood-red color while the water layer was deep green. The extraction with ether was continued until the water layer was no longer green. The combined ether extracts were evaporated in a desiccator without heat. A black, tar-like solid was left very much like the original material but it had the ester odor. It was partly soluble in water and readily soluble in alcohol. The alcoholic solution was tested on the skin and found to be not poisonous. The ester, or mixture of esters, was not investigated further in this connection, but was later shown to give the reactions for gallic acid and methyl furfurol. These reactions will be referred to in connection with other experiments.

A few other preliminary experiments showed that the original mixture was very complex and that it would be necessary to fractionate it and examine the fractions separately. Chlorophyll could not be removed because the solvents for chlorophyll, alcohol, ether, carbon bisulphide, etc., dissolve large quantities of the mixture.

Some of the original material was extracted with 50 per cent. alcohol, filtered from insoluble tar, and the following experiments were performed with the filtrate, F.

(1) A portion was distilled under diminished pressure, and a yellow, acid liquid was obtained. This was shaken with ether and the ether was evaporated. A small quantity of a yellow solid was left. The substance was not volatile enough to justify the use of this method for getting it.

(2) A precipitate obtained by adding lead acetate in 50 per cent. alcohol to a portion of the filtrate, F, was suspended in water, decomposed by hydrogen sulphide, shaken with ether, and the ether evaporated The residue, after drying in a desiccator, was a yellow solid, apparently the same as that obtained by distillation in (1) above.

(3) Another portion of the alcoholic filtrate, F, was filtered through bone-black. The colorless filtrate was apparently pure alcohol and water, everything in solution having been removed by the bone-black.

In precipitating an alcoholic solution of the crude material with a solution of lead acetate, it was noticed that at least two kinds of precipitates were formed. The part that went down first was darker in color than that thrown down later. Pfaff used the last fractions in obtaining his oil and stated that these precipitates consisted of the lead compound of the oil in a pure state. It was found by experiment that the darker part was soluble in ether while the lighter part was not. This indicated that the darker part consisted of tarry matter which was brought down mechanically, or separated out when the alcoholic solution was diluted by the lead acetate solution, or was perhaps a lead compound, soluble in ether. To test this point an experiment was made as follows: Some of the crude material was thoroughly extracted with 50 per cent. alcohol. The tar insoluble in 50 per cent. alcohol, was then treated with 95 per cent. alcohol. Most of it dissolved; the solution was filtered and lead acetate in 50 per cent. alcohol was added. A greenish colored precipitate was formed which was filtered off and found to be completely soluble in ether and soluble to a considerable extent in strong alcohol.

These experiments suggested that the light-colored lead compound which was thought to contain the poison could be purified by extraction with ether in a Soxhlet apparatus more conveniently than by the tedious process of fractional precipitation. Further preliminary experiments showed that 50 per cent. alcohol extracted from the original material all of the substance or substances which gave the light-colored precipitate and dissolved only a small amount of the tar.

Some of the crude material (288 grams) was then extracted several times with 50 per cent. alcohol and filtered; the insoluble tar was washed and saved for examination. To the filtrate was added an excess of a solution of lead acetate in 50 per cent. alcohol. The large precipitate, which will be designated as "Precipitate A," was filtered and drained by suction in a Büchner funnel. The alcoholic filtrate, "A," was saved.

Precipitate A was extracted with ether in Soxhlet extractors until the ether came over practically colorless, the operation being interrupted from time to time to stir up the precipitate in the thimble. The green-colored ether solution was saved for future examination. The lead precipitate, after extraction with ether and drying, weighed about 116 grams. A portion of this lead compound was decomposed by hydrogen sulphide in a mixture of water and ether which was well shaken during the operation. The ether was separated, filtered, and evaporated under diminished pressure without heat, and there remained a yellow oily-looking residue having a pleasant odor. When the ether and water were completely removed in a vacuum desiccator, a hard, brittle, yellow resin weighing about 16 grams was obtained. It was soluble in alcohol, had a strong acid reaction, and was free from nitrogen[1] and sulphur. The nitrogen tests were made by the Lassaign and soda-lime methods,[2] and the sulphur test was made with sodium nitroprusside after fusing the residue with sodium. The main portion of the lead compound was decomposed under alcohol by hydrogen sulphide, filtered, and the alcoholic filtrate evaporated *in vacuo*. The same yellow acid resin was obtained. Experiments continuing through several weeks were made in applying solutions of this resin to rats, rabbits, and guinea-pigs. Finding it to be without effect upon these animals, it was tried on Dr. Syme and found to be not poisonous.[3] In the meantime the resin was being studied in the laboratory.

GALLIC ACID.

An alcoholic solution of the resin was just neutralized with potassium hydroxide. During the titration, the solution rapidly became dark brown. After neutralization it was shaken with ether; the water solution remained brown while the ether layer

[1] Nitrogen was found very readily by the soda-lime test in the tar left after extracting the original material with 50 per cent. alcohol, but was not found by the Lassaign test.

[2] Stevens, *Amer. Journ. of Pharm.*, lxxvii, p. 255, 1905.

[3] Whenever it is stated in this paper that a solution was poisonous or not poisonous, the test was made by Dr. Syme upon himself.

was nearly colorless and contained practically no dissolved substance. A portion of the water solution of the potassium salt on being acidified with sulphuric acid and standing over night, deposited a slight precipitate with lead acetate somewhat similar to the original lead precipitate, A, and also slight precipitates with salts of zinc, mercury, copper, and silver (with reduction). It gave a bluish-black color with impure ferrous sulphate and a dark color with ferric chloride. It reduced ammoniacal silver nitrate and Fehling's solution. These experiments indicated the presence of a tannin compound. An alcoholic solution of the resin gave the same color reactions with iron salts as did the potassium salt.

Further tests with a solution of the resin in dilute alcohol, and with a water solution of the acid precipitated by adding sulphuric acid to a solution of the resin in potassium hydroxide led to the conclusion that the acid is gallic acid. These tests were the following:

(1) Boiling with an excess of potassium hydroxide gave a black substance (tauromelanic acid).

(2) The acid was not precipitated by gelatin.

(3) On addition of potassium cyanide a transitory red color appeared which reappeared on shaking with air.

Gallic acid is distinguished from tannic acid by tests (2) and (3).

At later stages in the work the potassium, barium, and sodium salts of gallic acid were obtained, and finally the pure acid was made by decomposing the sodium salt with sulphuric acid and crystallizing from water. The melting-point behavior of the acid corresponds with that of gallic acid; it melted with decomposition at about 230° C.

For further identification, some of the acid was converted into the ethyl ester by the method of Grimaux.[1] The ester obtained agreed in melting point and other properties with the ester described by Grimaux. The ester was also made from gallic acid from another source and the esters agreed in properties. A mixture of the two esters melted within the limits given for the ethyl ester of gallic acid (156°–159°C.).

[1] *Bull. de la soc. chim.*, ii, p. 95, 1864.

While the tests leading to the identification of gallic acid were being made, another series of experiments was in progress. Eleven and one-half grams of the resin obtained from lead precipitate, A, by decomposition with hydrogen sulphide were treated with $\frac{N}{10}$ potassium hydroxide added from a burette until the acid was exactly neutralized. All went into solution. On shaking with ether some of the potassium salt separated out and was saved for examination. The solution became dark brown on exposure to air and grew darker as the work proceeded. The acid in solution as a potassium salt was precipitated out in four fractions by adding for each fraction one-fourth the amount of $\frac{N}{10}$ sulphuric acid required to neutralize the potassium hydroxide used.

The precipitates were filtered off and examined. The first was small in amount, gummy, and hard to filter. The solution was shaken with ether after each precipitate had been filtered off. The succeeding precipitates were in better condition, but were not pure. All appeared to be impure gallic acid which had become brown by absorption of oxygen. They were saved, however, to be tested for poison. After the last fraction had separated, the filtrate was shaken several times with ether and saved for further examination, which will be described under "Rhamnose." This filtrate is designated as "B."

A portion of the resin obtained from the lead precipitate, A, was tested and found to be not poisonous as already mentioned. By this test, all the substances contained in the lead precipitate, A, after its extraction with ether in the Soxhlet apparatus, were eliminated from the possible poisonous substances. The poison must, therefore, have been extracted by the ether.

A portion of the original poisonous material was treated with 50 per cent. alcohol and filtered from insoluble tar. The filtrate was precipitated in six fractions by lead acetate. The last fractions were lighter in color and apparently much purer than the first. The sixth lead precipitate was decomposed by hydrogen sulphide. The light yellow water solution was tested and found to be not poisonous. It gave the characteristic reactions for gallic acid. The poison, if precipitated at all by lead acetate, must have gone down in one of the preceding fractions. Later

experiments showed that it is brought down partly mechanically and partly as a lead compound in the first precipitation.

FISETIN.

Some of the original material was extracted with hot water to remove gallic acid and filtered from tar while hot. The filtrate had a deep yellow color. On cooling over night, an olive-green precipitate separated out which was dried and found to be a light powder. It was practically insoluble in cold water, soluble with great difficulty in boiling water from which it separated in yellow flakes, slightly soluble in ether and in acetic acid, but readily soluble in alcohol. The solutions were not acid to litmus, gave a dark color with ferric chloride, an orange-red precipitate with lead acetate which was easily soluble in acetic acid, and an orange yellow precipitate with stannous chloride. These properties and reactions indicated that the substance was the dye-stuff, fisetin, and that it occurs in the free state in this plant though it is usually found as a glucoside of fisetin combined with tannic acid.

The substance which separated from the boiling-water solution was further purified by dissolving it in a small quantity of hot water. On cooling, the yellow compound separated out in a flocculent condition. Examined under a microscope, the flakes appeared to be made up of masses of fine crystals. The substance was found to have the characteristic properties of fisetin described by Schmid,[1] and on fusion with caustic potash,[2] gave protocatechuic acid and phloroglucinol. These substances were identified by characteristic tests.

RHAMNOSE.

Schmid[3] obtained a sugar solution by the decomposition of a fisetin-glucoside from *Rhus cotinus*, and Perkin[4] obtained the same from a glucoside in *Rhus rhodanthema*. They thought

[1] *Ber. d. deutsch. chem. Gesellsch.*, xix, p. 1740.
[2] *Ibid.*, p. 1747; *Ann. d. Chem.* (Liebig), cxii, p. 97.
[3] *Loc. cit.*, p. 1738.
[4] *Journ. Chem. Soc.*, lxxi, p. 1194

that the sugar in both cases was isodulcite, but they did not isolate it. Assuming that the free fisetin found in *Rhus toxicodendron* had its origin in the decomposition of a fisetin glucoside by natural processes, it was reasonable to suppose that the sugar would also be found in the free state, although with one or two possible exceptions,[1] rhamnose has not been found except as a glucoside body.

Since rhamnose forms a lead compound, the sugar, if present, should be found in the first lead precipitate, A, and also in the filtrate, A, in case it is not completely precipitated in the presence of acetic acid and alcohol.

The filtrate, A, (about two liters) was examined first. It had a light yellow color, contained an excess of lead acetate, and was acid from the acetic acid liberated in the precipitation of the lead compound, A.[2] This filtrate was evaporated to dryness under diminished pressure to remove alcohol, water, and acetic acid. The clear distillate had a peculiar odor suggesting both tea and amyl formate. It was saved for examination and was found to be not poisonous. The residue in the dish after evaporation was a tough, reddish-brown, gummy mass which could be drawn out into fine threads. It had a pleasant sweet odor. It was extracted several times with hot water, each portion being filtered. A yellow-brown powder remained undissolved and was saved. The combined filtrates deposited more of the yellow solid on standing. The filtered liquid was freed from lead by hydrogen sulphide. The solution then had a lemon-yellow color, a sweet odor, and was acid from acetic acid. On concentrating the solution by evaporation and making a small portion of it alkaline with sodium hydroxide, the yellow color became very intense.[3] The alkaline solution reduced Fehling's solution and ammoniacal silver nitrate, indicating the presence of a sugar. Another portion of the solution gave a slight precipitate with

[1] *Chem. Zeitung*, xxiii, Ref. p. 177.

[2] On standing several weeks, a small quantity of tar separated out on the walls of the vessel; also a brown precipitate which was filtered off, suspended in water, and hydrogen sulphide was being passed in, when an accident occurred and it was lost.

[3] "By warming with alkalies or barium hydroxide, rhamnose is colored yellow."—*Chemie der Zuck.*, i, p. 177, 1904.

phenylhydrazin in the cold. The remainder of the solution was evaporated to dryness, extracted with water, filtered, and again evaporated. A dark sticky syrup was left which was only partly soluble in water. This was treated with water, the filtrate filtered and evaporated, the water being replaced from time to time to remove acetic acid. Finally the liquid gave the following tests for rhamnose, besides those already mentioned:

(1) With α-naphthol[1] and sulphuric acid, a purple violet color.

(2) With thymol[2] and sulphuric acid, a red color.

(3) With resorcinol[3] and sulphuric acid, a red color.

(4) With orcinol[4] and hydrochloric acid, a red color.

(5) With ammonium picrate and sodium picrate, a yellowish red color.

(6) With phloroglucinol and hydrochloric acid, a red color.

(7) It decolorized an alkaline solution of potassium ferricyanide.

(8) It gave a white precipitate with lead acetate.

The filtrate, B, (p. 553) from which gallic acid was precipitated by sulphuric acid in four fractions was saved to be examined for sugar. To remove gallic acid completely, and other vegetable matter, it was shaken out several times with ether, and was kept at a low temperature with salt and ice for a long time. It was left standing for several weeks, during which time more brown matter separated out and was filtered off. The filtrate was evaporated to a small bulk, cooled, and filtered from crystals of potassium sulphate. The filtrate was evaporated to dryness, the residue taken up in water and filtered through bone-black. Addition of alcohol caused complete precipitation of potassium sulphate. The solution then gave the above mentioned characteristic tests for rhamnose.

All attempts to get the osazone of the sugar by the method of Fischer failed, probably on account of the small quantity of sugar present. The plant, it will be remembered, was origin-

1 *Chem. der Zuck.*, i, p. 188, 1904.
2 *Ibid.*
3 Rayman, "Sur l'Isodulcite," *Bull. de la soc. chim.*, xlvii, p. 668, 1887.
4 Garros, *Acides Gummiques.*

ally extracted with ether in which rhamnose is practically insoluble. The above described tests, however, can leave no doubt as to the identity of the sugar.

Additional evidence that the sugar is rhamnose was obtained by a method for testing for the presence of this sugar described by Maquenne.[1] This method consists in converting the sugar into methyl furfurol by distilling the substance to be tested with dilute sulphuric acid. The presence of methyl furfurol in the distillate is shown by a green color with alcohol and sulphuric acid. "This procedure is applicable to extracts as well as to entire plants, and has the advantage that it does not require the separation of isodulcite, the crystallization of which is often very slow, and at times impossible, when it is mixed with other very soluble substances."[2]

The experiment was tried with the crude ether extract of the plant according to the direction of Maquenne, and the green color with alcohol and sulphuric acid was obtained from the thicker oily portion of the distillate. This test can be made with hydrochloric acid[3] as well as with sulphuric acid. Therefore, the color test was tried with the ester mixture prepared in one of the early experiments by boiling the original plant material with hydrochloric acid and alcohol. Methyl furfurol was found here also, this method indeed giving better results than that of Maquenne.

The presence of free rhamnose has thus been shown in the original material, in the first precipitate by lead acetate, and in the filtrate from this precipitate. Experiments to be described under "The Poison" showed that the ether extract from the Soxhlet apparatus contained a substance which yielded rhamnose when hydrolyzed by dilute sulphuric acid.

The presence of free gallic acid, fisetin, and rhamnose in the plant can readily be explained by a series of assumptions for which there is a considerable amount of experimental evidence. There is reason to believe that tannin-like bodies are formed at the expense of chlorophyll,[4] that complex tannin bodies can

[1] *Ann. de chim. et phys.*, xxii, p. 93, 1891.
[2] *Ibid.*
[3] *Biochem. d. Pflanzen*, i, p. 210.
[4] *Compt. rend. de l' Acad. des sci.*, cxv, p. 892.

be broken down by acetic acid (also found in *Rhus toxicodendron*) into a tannic acid and a glucoside (for example, "fustin-tannide"[1] yields tannic acid and fisetin glucoside); and finally that the glucoside can be hydrolyzed by acids or enzymes giving, in the sumach plants, fisetin and rhamnose.

Nitrogenous ferments which can effect the hydrolysis of glucosides and give rise to sugars are frequently found in plants, for example, emulsin in almonds, myrosin in mustard, and erythrozym in madder. Acree and Hinkins[2] found that astase, pancreatin, and a number of other enzymes cause the hydrolysis of triacetyl glucose with the formation of glucose and acetic acid. Stevens[3] obtained a nitrogenous oxidizing enzyme from *Rhus vernicifera*. The close relationship between the poisonous species of *Rhus* would lead us to suppose that the same soluble ferment exists in poison ivy, though it was not detected in the original material used in these experiments, probably because the plant was extracted with ether in which the enzyme is insoluble. The existence of such a soluble ferment would explain the presence of free sugar and free fisetin.

THE POISON.

Some of the original poisonous material (288 grams) was extracted with 50 per cent. alcohol, and this alcoholic solution was precipitated with lead acetate in the manner already described (p. 550). The lead precipitate so obtained was extracted with ether in Soxhlet extractors and after the extraction was found by test to be free from poison. Therefore the poison, if precipitated by the lead acetate, must have been extracted by the ether. This ether solution had a dark green color, and was acid from acetic acid brought down in the lead precipitate. The ether was evaporated in a vacuum desiccator without heat and there remained a small quantity of an acid mixture of water and a soft tar; the watery part was colored green showing that the tar was soluble to some extent in dilute acetic acid. The mixture had the peculiar odor of the original material.

1 Schmid, *loc. cit.*, p. 1736.
2 *Amer. Chem. Journ.*, xxviii, p. 370.
3 *Amer. Journ. of Pharm.*, lxxvii, p. 255, 1905; lxxviii, p. 53, 1906.

A small drop of the green watery part was applied to the wrist, allowed to remain a few minutes and was then removed by absorbent paper, but the spot was not washed. Itching and reddening of the skin commenced within twenty-four hours. At the end of forty-eight hours there was a well developed case of poisoning. This was cured by immersing the arm in a hot solution of potassium permanganate.

A small portion of the poisonous mixture was dissolved in alcohol, and this solution was divided into three parts. The first part was treated with ferric chloride but it gave no color reaction. Another portion of the alcoholic solution was diluted with water. It became turbid. The third portion gave a dirty-green precipitate with lead acetate, which seemed to come down more readily when the solution was diluted with water. The main portion of the poisonous mixture was then dissolved in 95 per cent. alcohol, and lead acetate in 50 per cent. alcohol was added. The precipitate was filtered, washed, and decomposed by hydrogen sulphide in a mixture of water and ether. The ether solution was filtered and evaporated. The residue was a tar which on standing in a desiccator for some time became dry enough to break into sticky lumps. An alcoholic solution of this substance gave a dark color with ferric chloride and a light-colored precipitate with lead acetate.

To get more of the poisonous tar for study, 233 grams of original material were extracted with 95 per cent. alcohol. Strong alcohol was used in order to dissolve as much of the tar as possible. The solution had a dark greenish color but was somewhat yellow in thin layers. The undissolved tar was filtered off and extracted twice again in the same way. The tar left after the third extraction was only slightly soluble in alcohol, and its solution was not poisonous. The three filtrates from these three extractions were precipitated separately by lead acetate in 50 per cent. alcohol. The first precipitate was largest, darkest in color, and carried down the most tarry matter. The second was light green. The third, which was quite small, was black, and was not a lead compound at all, but some of the tar which separated out on diluting the strong alcohol with the weaker containing lead acetate. It was soluble in ether and less soluble in alcohol. The alco-

holic solution of this third lot gave no precipitate with hydrogen sulphide. The first and second lead precipitates were filtered by suction and washed with water. They were kept a day or two in a desiccator over sulphuric acid but did not become completely dry. The weight of these two moist precipitates together was 172 grams. They were combined and extracted with ether in Soxhlet extractors which were kept in operation during work hours for three days.

In the meantime the alcoholic filtrates from these lead precipitates were combined and concentrated on the water-bath by distilling off two liters of alcohol. The alcohol obtained had the peculiar odor of the original material, but was not poisonous.

After a long extraction of the lead precipitate in the Soxhlet extractors, the green ether solutions were combined and washed by shaking them with water to remove lead acetate and acetic acid in case any should have been held in the lead precipitate. The ether was distilled off at a low temperature and there remained a soft tar, a portion of which was not completely soluble in 95 per cent. alcohol. The alcoholic solution had a greenish-yellow color and was poisonous. The tar was also partly soluble in acetic acid, and this solution was found to contain lead. Thinking that the lead acetate had not been completely washed out, the main part of the tar was dissolved in ether and shaken with water. The wash water continued to give a test for lead as long as the washing was continued. This indicated probably the hydrolysis of an unstable lead compound. Hydrogen sulphide was passed into the ether solution mixed with water to remove the lead. Lead sulphide was filtered off, and the ether was evaporated. A small portion of the tar residue in alcoholic solution gave a color reaction with ferric chloride. As this may have been due to traces of lead gallate dissolved in the extraction with ether and afterwards decomposed by hydrogen sulphide, the main portion of the tar was redissolved in ether and shaken with water until it no longer reacted with ferric chloride. The ether was then evaporated and a soft, black, poisonous tar or gum of uniform consistency was left which was shown by tests to be free from gallic acid and lead These experiments showed that some of the poison was precipitated as a lead compound, soluble in ether, and some wa

brought down mechanically in the free state. To see if the extraction with ether in the Soxhlet apparatus was complete, the residue in the thimbles was decomposed by hydrogen sulphide and shaken with ether. The dark-colored ether solution was freed from gallic acid by shaking with water and dilute sodium carbonate solution, and was evaporated. A small quantity of tar was obtained which was added to the main portion.

A solution of the poisonous tar in 95 per cent. alcohol did not reduce Fehling's solution and did not give a precipitate with lead acetate except the separation of a small quantity of tar, which was not a lead compound. The lead compound of the poison was apparently soluble in 95 per cent. alcohol as well as in ether, for it would not precipitate in this medium, although it was found in the original precipitate by lead acetate. The alcoholic solution of the tar became turbid on diluting with water.

In order to see if the poison is volatile with vapor of acetic acid, since this acid is found in the plant and it is thought by some that the poison is volatile, a portion of the tar was distilled under diminished pressure with acetic acid. It was soluble to some extent in the acid. The temperature did not go higher than 55°C. during the distillation. A tube containing cotton wet with sweet oil was placed between the receiver and the water suction so that the uncondensed vapors would have to pass through the cotton. This cotton was rubbed on the skin and was not poisonous. The yellow distillate collected in the receiver was also tested and was not poisonous.

HYDROLYSIS OF THE POISON.

About 5 grams of the tar, free from gallic acid and sugar, were dissolved in alcohol, and dilute (2 per cent.) sulphuric acid was added. Some of the tar separated out on diluting the alcohol with the acid. The mixture was heated on a water-bath during work hours for four days. A purple and green fluorescent solution was formed, though much tar was left apparently unchanged. The alcohol was evaporated off and the solution was filtered from tar. The fluorescent filtrate was shaken with ether, by which the green substance was removed, leaving

the solution purple. The ether left, on evaporation, a small quantity of a green substance having a pleasant ester odor. It was not further examined. A portion of the purple solution was exactly neutralized with sodium carbonate. This solution gave a blue-black color with ferric chloride, which became red on addition of another drop of sodium carbonate, indicating gallic acid. It also reduced Fehling's solution.

Another portion of the purple solution was made alkaline with sodium carbonate. A reddish-brown flocculent precipitate was formed and was filtered off. The filtrate did not give any color with ferric chloride, but it reduced Fehling's solution. It also gave the test for rhamnose with α-naphthol.

The main portion of the purple solution was made alkaline with sodium carbonate; the precipitate was filtered off and dissolved in acetic acid. The solution was yellow and gave a reaction with ferric chloride similar to that of gallic acid. The filtrate from the precipitate by sodium carbonate was concentrated by evaporation until sodium sulphate began to crystallize out. Alcohol was added to precipitate the sodium sulphate completely; the mixture was heated and filtered. The alcoholic filtrate was concentrated to a syrup which reduced Fehling's solution and gave the characteristic tests for rhamnose already described. By this hydrolysis, the tar was split up into rhamnose and some form of gallic acid which could be precipitated by sodium carbonate. This compound, whose acetic acid solution was yellow, probably contained fisetin also. The reason for this last statement will appear from the following experiment.

DECOMPOSITION OF THE POISON WITH ACETIC ACID.

A portion of the poisonous tar was heated in an open dish with strong acetic acid. The tar seemed to be decomposed to some extent, giving a yellow substance. Acetic acid was added from time to time as it evaporated. After several evaporations, water was added, the mixture was heated to boiling and filtered. This filtrate, No. 1, will be mentioned later. The residue in the dish consisted of undecomposed tar and an olive-green, flaky substance. This substance was heated with a fresh

portion of glacial acetic acid. Water was added, and the mixture was boiled and filtered. The filtrate had a deep yellow color suggesting fisetin. It was shaken out with ethyl acetate which became colored yellow. A portion of the ethyl acetate solution gave an orange-red precipitate with lead acetate, showing the presence of fisetin. The ethyl acetate was removed from the remainder of the solution by evaporation and the yellow residue was taken up in alcohol. This alcoholic solution gave the characteristic reactions for fisetin with stannous chloride, with potassium hydroxide, with ferric chloride, and with Fehling's solution.

Filtrate No. 1, obtained by heating the poisonous tar with acetic acid and hot water as described above, was investigated as follows: A portion of it gave a reddish-colored precipitate with sodium carbonate as in the case when the tar was hydrolyzed with sulphuric acid. The remainder was nearly neutralized with sodium carbonate and lead acetate was added in excess to remove gallic acid. The excess of lead was removed by sulphuric acid, and the sulphuric acid was removed by barium carbonate. The solution on evaporation reduced Fehling's solution to some extent, but a white precipitate was also formed.

In this experiment, gallic acid and fisetin and probably sugar were formed by decomposition of the poisonous gum with acetic acid, the acid found in the plant by Pfaff. The presence of free gallic acid, fisetin, and rhamnose in the plant can be explained by the natural hydrolysis of a complex gum or tar or a constituent thereof.

The poisonous property is lost in the general rearrangement which takes place during hydrolysis.

The poisonous tar was not hydrolyzed by boiling with a dilute solution of sodium carbonate.

It was found, as has been stated elsewhere, that the lead compound of the poison could not be precipitated in 95 per cent. alcohol. Further experiments, however, showed that on extracting the poisonous gum with 50 per cent. alcohol, a portion of it dissolved, and this solution gave a precipitate with lead acetate which was a true lead compound. The remainder of the purified tar (about 10 grams) was treated with

50 per cent. alcohol and filtered. Very little dissolved in alcohol of this strength, but on addition of lead acetate, in 50 per cent. alcohol, to the solution, a light-colored precipitate was formed, which became dark on standing. It was filtered off, washed free from lead acetate, decomposed by hydrogen sulphide, and shaken out with ether. The ether left, on evaporation, a yellow resinous substance having a faint odor like garlic. By drying in a desiccator, a small quantity of solid yellow resin was obtained which was completely soluble in alcohol. A very small drop of this solution applied to the skin on the end of a glass rod which had been drawn out to a point caused an eruption in about thirty-six hours. Following the nomenclature used by Maisch and Pfaff, this substance will be designated as *Toxicodendrin*, the ending "in" indicating its glucoside nature.

The filtrate from the lead precipitate just described was freed from the excess of lead acetate by hydrogen sulphide, was tested for poison, and was found to be poisonous, showing that the precipitation by lead acetate was not complete even in 50 per cent. alcohol. On spontaneous evaporation of the solution, a yellow, sweet-smelling resin was left.

A portion of the alcoholic solution of the toxicodendrin gave a dark coloration with ferric chloride, did not reduce Fehling's solution, and was slightly acid to litmus.

To see whether the toxicodendrin could be hydrolyzed, the remainder was dissolved in alcohol and dilute sulphuric acid was added. A fine white precipitate was formed at once which rose to the surface on standing as a light flocculent substance. The mixture was heated for several days on a water-bath, filtered from unhydrolyzed resin, and the filtrate was neutralized and concentrated in the way already described. The solution obtained reduced Fehling's solution. Not enough was obtained for further sugar tests, but all the hydrolysis experiments point to the conclusion that the poisonous substance is a rhamnoside, and is the source of the sugar in the plant.

The reaction with ferric chloride, observed whenever a lead compound of the poison is decomposed by hydrogen sulphide, may be explained by the formation of traces of gallic acid or fisetin through the action of the weak acids present.

The supply of purified poisonous tar having been exhausted

in the preceding experiments, further study of the active principle is postponed until more can be prepared. It is highly desirable to investigate the white precipitate formed by addition of sulphuric acid to an alcoholic solution of the toxicodendrin.

OXIDATION OF THE POISON WITH NITRIC ACID.

When the purified poisonous material (p. 556) was extracted with 50 per cent. alcohol, only a small quantity was dissolved, as was stated above. The insoluble residue was treated with fuming nitric acid. Violent reaction took place at once with copious evolution of red fumes and heat. When the reaction was over, a sticky red gummy mass was left which was slightly soluble in warm alcohol. The water extract was yellow, and the alcoholic solution was red. That the water extract contained picric acid was shown by the following experiments:

(1) A portion was gently warmed with a few drops of a strong solution of potassium cyanide and two drops of sodium hydroxide. The red color of potassium isopurpurate was formed.

(2) A portion of the water solution was heated with glucose and a few drops of sodium hydroxide. The deep red color of picraminic acid was produced.

(3) A few drops of an ammoniacal solution of copper sulphate were added to the water extract. A yellow-green precipitate was formed.

(4) The water extract dyed silk, but did not dye cotton cloth.

DISTILLATION OF THE TAR WITH SODA-LIME.

About 25 grams of the tar left after extracting the original material with hot water were dissolved in ether and poured into a glass retort containing soda-lime. The ether was distilled out, leaving the tar intimately mixed with the soda-lime. The retort was then gradually heated. Vapors and liquid were given off, both of which turned red litmus blue and had a strong odor like tobacco smoke. No odor of ammonia was detected.[1] At the high temperature of the triple burner, a semi-solid, red,

[1] See *Amer. Journ. of Pharm.*, lxxvii, p. 256.

greasy substance collected in and closed the condenser tube. This substance had the same powerful odor as the liquid portion of the distillate. The clear, watery portion of the distillate was separated from the thicker parts, and was found to contain pyrrol and pyridin derivatives by the following characteristic tests:

(1) Wood moistened with hydrochloric acid was turned red by it.

(2) Colorless fumes were formed when brought near hydrochloric acid; mixed with hydrochloric acid, a red insoluble substance was formed.

(3) It precipitated the hydroxides of iron, gave a light blue precipitate with copper sulphate, and a white precipitate with mercuric chloride.

The greasy, semi-solid mass was extracted with 10 per cent. hydrochloric acid and filtered. On addition of a solution of mercuric chloride to the red filtrate a brown flocculent precipitate was formed. It was filtered off and distilled with caustic soda, but the distillate did not contain pyridin.

OXIDATION OF THE NON-POISONOUS TAR WITH NITRIC ACID.

Eight grams of the neutral tar, from which the poison had been extracted by means of 50 per cent alcohol, was heated with 20 c.c. of concentrated nitric acid for a few hours. The black tar turned yellow, with much foaming and with the evolution of great volumes of oxides of nitrogen. When the nitric acid was nearly all evaporated 25 c.c. of fuming nitric acid was added. Again a very vigorous action took place. The mixture was heated several hours and the nitric acid was poured off. The residual gum (A) was extracted several times with hot water and the extracts were combined. These water extracts were shaken out several times with ether. The combined ether extracts were evaporated to a small volume and this was allowed to evaporate spontaneously. The ether left a residue of a mixture of yellow crystals of picric acid (B), and of white crystals of another acid (C). These crystals were separated mechanically.

The picric acid (B) formed a red ammonium salt and gave the following tests:

1. It dyed wool, but not cotton.

2. A portion was gently warmed with a few drops of a strong solution of potassium cyanide and three drops of 10 per cent. potassium hydroxide solution. The red color of potassium isopurpurate was formed.

3. A portion was heated in water solution with glucose and a few drops of 10 per cent. sodium hydroxide solution. The deep red color of picraminic acid salts was formed.

4. A few drops of an ammoniacal solution of copper sulphate were added to a water solution of the picric acid. A yellow-green precipitate was formed.

The white acid (C) was dissolved in sodium hydroxide solution and to this a solution of hydrochloric acid was added slowly. A precipitate of the acid was formed, which, when dry, melted unsharply at 100°–105° C. This acid is soluble in hot water and gives a yellow precipitate with ferric chloride solutions, but not a dark color. It does not reduce Fehling's solution, nor does it give the red color of picraminic acid salts when warmed in water solution with glucose and sodium hydroxide.

The gum (A) was treated with ether and sodium hydroxide solution in a separatory funnel to remove any acids from the gum. The alkaline layer turned dark brown; it was separated from the ether, warmed to remove the dissolved ether, and acidified. A precipitate of about 3 grams of a solid acid was formed. This acid was dissolved in 50 c.c. of absolute alcohol containing 2 grams of hydrochloric acid and the solution was boiled three hours under a return condenser to convert the organic acid into an ester. This alcoholic ester solution was poured into 200 c.c. of water, and the mixture was shaken out several times with ether to remove the ester. The ethereal solution of the ester was shaken out several times with 10 per cent sodium hydroxide solution to remove any acids, and was then separated and dried with anhydrous sodium sulphate. The ether was distilled off and the residue of esters was fractionated at a pressure of 18 millimeters. One fraction, about 2 grams, distilled at 150°–170° C. and another, about 0.5 gram, came over at 170°–190° C. Both of these fractions had a delightful ester odor, and both gave precipitates of potassium salts when they were saponified by hot alcoholic potassium hydroxide. These esters and their acids will be investigated further.

HYDROLYSIS OF THE NON-POISONOUS TAR WITH ALCOHOLIC *POTASSIUM HYDROXIDE.*

Some of the neutral tar (78 grams) was boiled 48 hours with the same weight of potassium hydroxide in sufficient 90 per cent. alcohol to dissolve the solids. The solution was then evaporated in a dish to remove all of the alcohol, and the residue remained undisturbed several months. The residue was then warmed for some time with 500 c.c. of water to dissolve any potassium salts and was filtered. The very dark filtrate was shaken out with ether and by this means a large amount of tarry matter was removed from the alkaline solution. The water solution was separated and warmed to remove the ether, and was then acidified. A copious green-black precipitate (A) was formed and was filtered off. The filtrate had a fine blue-green color by reflected light and a purplish-green fluorescence by transmitted light. When part of this blue-green solution was shaken out with ether, part of the substance (B) was extracted. The main portion of the solution was evaporated to a small volume and cooled. A small precipitate (C) was formed which was apparently identical in color and behavior with the acid (A). This small precipitate was soluble in hot water and in ether and formed a blue-green solution in both solvents. The chief green-black acid (A) was redissolved in sodium hydroxide solution and precipitated by sulphuric acid. It came out again as a green-black solid which was filtered; the filtrate was colored blue-green as above. The precipitate was soluble in hot water, alcohol, and ether and formed blue-green solutions.

The green substance in the filtrate from C and the substance in the ether extract (B) gave identical color tests as the following experiments will show:

The substance (B) was dissolved in sodium hydroxide and formed a brownish solution which was used for the following tests:

1. The alkaline solution (B) was not changed in color when heated with glucose or potassium cyanide.

2. Nitric acid, when added to an equal volume of the salt solution (B), gave at first a blue-green color, which changed to rose-red when heated, and the solution finally became colorless.

3. When hydrochloric acid was added to the alkaline solution (B), the color changed to blue-green, which did not change on boiling. When a small piece of potassium chlorate was added to the hot solution, the color became rose-red and finally the solution became colorless.

4. When hydrochloric acid and zinc dust were added to the alkaline solution (B), the green color disappeared when the mixture was heated some time. The colorless solution was treated with a small piece of potassium chlorate and heated; the colorless solution became rose-red and afterwards colorless. This colorless solution was again reduced with zinc dust and hydrochloric acid at boiling temperature and the zinc dust was removed by filtration; the filtrate was again treated with two drops of hydrochloric acid and a grain of potassium chlorate and heated, but no rose-red color appeared.

5. When some of the alkaline solution (B) was treated with a small crystal of potassium permanganate, the solution turned green and manganese dioxide precipitated out. The solution was then made acid with acetic acid and boiled with some alcohol; a colorless solution resulted.

6. Some of the alkaline solution (B) was treated with hydrochloric acid and a small crystal of potassium bichromate was added and the solution boiled. The color changed from green to dark rose-red and then to the yellowish red of acid chromate solutions.

7. When some of the alkaline solution (B) was heated with hydrogen peroxide solution, no change in color took place; but when this solution was treated with hydrochloric acid and warmed, the color became faint rose-red and afterwards light green. The light green color did not disappear on boiling, but did so when a small piece of potassium chlorate was added, as above in 3.

8. Some of the deep green mother-liquor (C) was warmed with nitric acid; it turned deep claret at first and then became lighter and finally colorless, as in 2. The substances (B) and (C) behave alike in this reaction.

9. Some of the concentrated solution (C) was treated with hydrochloric acid and a small crystal of potassium chlorate; the solution became deep claret in color when warmed and afterwards colorless, as in 3.

POTASSIUM PERMANGANATE AS A REMEDY FOR *RHUS* POISONING.[1]

In the early stages of this work some experiments were made to see if potassium permanganate could be used to purify the lead precipitate by oxidizing the tar brought down in precipitation. *It was found that the permanganate attacked the lead precipitate* as well as the other organic matter in the vessel. This fact and the well-known value of permanganate in treating skin diseases, its use as an antidote for some kinds of alkaloid poisoning,[2] as an antidote given to cattle poisoned by plants,[3] and as an antidote for snake bites,[4] suggested its use as a remedy for *Rhus* poisoning. Maisch[5] mentioned that he had used it with success, but it never came into general use, probably on account of its staining the skin and clothing. In carrying out this work abundant opportunities for testing its value as a remedy for the dermatitis caused by poison ivy were afforded by many cases of accidental and intentional poisoning. The best example of the latter was obtained with the ether solution from the extraction of the lead precipitate in the Soxhlet apparatus (p. 559). After removing the ether a small drop of the residue was applied to Dr. Syme's wrist as described. An itching red spot about the size of a dime was noticed in thirty-six hours, which steadily increased in size. Nearly two days after the application of the poison, a dilute solution of potassium permanganate containing a little caustic potash was rubbed into the spot until the pimples were destroyed. A little black spot was left wherever there had been a pimple, showing that the permanganate had been reduced to oxide in the skin. The place was washed and nothing more was thought of it until the morning following, when it was noticed that the wrist had commenced to swell during the night, and the characteristic watery secretion was running from the poisoned spot. More permanganate solution was applied without potash and the

1 This section is added in the hope that it may be of use to others who are subject to this form of poisoning.

2 Moor, *N. Y. Med. Rec.*, xlv, p. 200, 1894.

3 *Bull. No. 26, U. S. Dept. Agr., Div. of Bot.*, xlvii.

4 Lacerda, *Compt. rend. de l'Acad. des sci.*, xciii, pp. 466–469, 1881.

5 *Amer. Journ. of Med. Sci.*, lii, p. 285, 1866.

wrist was bandaged thinking that this would prevent the spreading of the eruption; but the bandage really facilitated the spreading by becoming saturated with the poisonous fluid and keeping it in contact with a larger surface of skin. In the meantime the swelling and inflammation had extended nearly to the elbow. The arm now had the appearance of having been bitten by a snake. To reduce the swelling it was immersed in hot water. This seemed to bring out the eruption very quickly and the blisters were treated with permanganate as fast as they appeared. The swelling was reduced, but returned during the night. On the evening following, the forearm was immersed in a bowl of hot permanganate solution containing a little caustic potash. The solution was kept as hot as could be borne for about half an hour. After this bath the poison was completely oxidized, for the swelling was reduced and did not return, nor was there any fresh eruption. What appeared to be a severe case of poisoning was thus cured very quickly. The use of hot water not only reduces the swelling but also helps to destroy the poison. The action of permanganate is also more rapid at high temperatures.

The oxidizing power of permanganate, as is well known, is greater in acid solution than in alkaline, five atoms of oxygen being available from two molecules of potassium permanganate in the former and three in the latter. Permanganate was used as a remedy, in some cases, mixed with dilute sulphuric acid, and in others, with zinc sulphate; also with lime water. It was found to be satisfactory whether used alone or with any of the substances mentioned, provided it was well rubbed into the skin. The concentration of the solution used was varied according to the location and condition of the eruption. Where the skin was thin or already broken, dilute solutions (1 per cent.) were used. In one case, the eruption appeared in the palm of the hand, where the skin was so thick that it was necessary to open it before the remedies could reach the poison. The difficulty of getting the remedy in contact with the poison in the skin is the reason why the eruption is hard to cure.

The remedy most commonly used for this eruption is an alcoholic solution of lead acetate. This remedy is unsatisfactory for the reason that its action consists in depositing an unstable

lead compound of the poison in the skin where the conditions of moisture and temperature are favorable for its decomposition, liberating the poison with all its irritant properties. Moreover, alcoholic preparations should not be used, because the alcohol dissolves the poison and, on evaporation, leaves it spread over a larger surface like a varnish. Potassium permanganate, however, oxidizes the poison completely. The only objection to the use of permanganate of which the writer is aware is that it stains the skin. The stain can be removed by vigorous scrubbing with soap, or it will wear off gradually in a few days. It can be removed at once by a warm solution of oxalic acid, sulphurous acid, etc., but these should not be used by persons not familiar with their action.

With the knowledge of the facts mentioned, many solutions were tested for poison by applying them to the skin, and when an eruption appeared, it was cured quickly and permanently by rubbing in a permanganate solution, usually mixed with dilute sulphuric acid.

SUMMARY.

Leaves and flowers of the poison ivy plant were extracted with ether and the ether was removed by evaporation. In the residue, the following substances were found and studied: gallic acid, fisetin, the sugar, rhamnose, and a poisonous tar, gum, or wax.

The lead compound of the poison is soluble in ether; this gives a means of separating the poisonous substance from the non-poisonous matter in one operation.

The poison is not volatile with vapor of acetic acid, or with vapor of alcohol.

The poisonous tar or wax was decomposed by acids and yielded gallic acid, fisetin, and rhamnose, showing the probable source of these compounds in the plants, and indicating that the poison is a complex substance of a glucoside nature.

It was found that a portion of the poisonous substance can be precipitated by lead acetate from a solution of the purified tar in 50 per cent. alcohol.

All cases of poisoning developed on Dr. Syme were easily cured with potassium permanganate.

The following method is suggested for obtaining the poisonous substance from the plant: Extract the plant with alcohol, filter, and precipitate at once with lead acetate. Wash the precipitate, dry, and extract with ether in Soxhlet extractors (loosely filled). Combine the ether extracts, mix with water, and pass in hydrogen sulphide. Separate the water and the ether solution, and filter the latter. Wash the ether solution thoroughly by shaking with water, and then evaporate at a low temperature.

THE ELIMINATION OF INDOXYL SULPHATE IN THE URINE OF THE INSANE.

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(Received for publication November 15, 1906.)

STATUS OF THE QUESTION.

An excessive production of indol in the intestine, manifested by increased elimination of indoxyl sulphate or indican in the urine occurs in a number of diseases. The causation of functional nervous disorders among others has been ascribed more than once to indol poisoning, and it is a fact of every-day laboratory observation that the urine of those suffering from migraine or "the blues" and that of persons even mildly neurasthenical frequently give, with the usual tests, a marked reaction for indoxyl.

Herter[1] discussed the question of the occurrence of indican and the paired sulphates in idiopathic epilepsy and concluded that attacks of convulsions were sometimes precipitated by an increase in intestinal putrefaction marked by an excess of indoxyl and other ethereal sulphates in the urine, and that these factors were most active in those who suffered from chronic intestinal catarrh, and generally occurred after excesses in eating. Under these conditions, he obtained by oxidation as high as 82 milligrams of indigo blue, determined gravimetrically, from the daily urine of a patient. The amount of indoxyl excreted varied widely, and might in the same subject be large one day and a bare trace the next.

Richardson[2] believed *indol* to be excessive in most forms of mental disease, and submitted a chart, including nearly all the functional insanities as well as some having an anatomical basis which tended to demonstrate this. He estimated indol by

[1] *N. Y. Med. Journ.*, August 20 and 27, and Sept. 3, 1892.
[2] *Bull. of the Lab. of the Mt. Hope Retreat*, 1889.

an original method, apparently quantitative, but not published.[1]

Pilcz[2] considered an increase as occurring in both phases of manic-depressive insanity and diminution in the elimination of indoxyl as certain evidence of the abatement of the psychosis whether of depressed or maniacal type.

In 1902, Coriat[3] devoted a lengthy paper and discussion to the study of the question of the excretion of indoxyl in the insanities. He concluded that, although "auto-intoxication, manifested physiologically by hyperindicanuria, has not been proved to be a factor in producing akinetic states, a striking parallelism exists between excessive indican elimination and various *akinetic* disorders, entirely independent of the form of mental disease," and "that *diminished* indican is parallel with catatonic excitement, the involution melancholias, the exhilarated forms of general paralysis." "In over one-third of manic cases, it was absent." Coriat drew a sharp distinction between cases with "hyperkinesis" or overactivity, and those of a depressed, inactive, or "hypokinetic character." He thought that gradual diminution to normal in the elimination of indoxyl preceded the clearing up of the depressive psychosis, and that the indican remained high until very evident improvement in the patient's condition could be noted.

In 1904, Folin[4] published the results of a carefully elaborated series of metabolism experiments on a large number of cases of insanity. He was of the opinion that his data, covering the results of practically complete urinary examinations in so extensive a series of insane cases of diverse type, proved "the untrustworthiness of all those metabolism experiments, old and new, which report a characteristic increase or diminution of any of the urinary constituents included in this research (*i. e.*, volume of urine, total nitrogen, urea, ammonia, uric acid, creatinin, organic bases, total sulphates, ethereal sulphates, neutral sulphur, phosphates, chlorides, organic or mineral acids, *indican*) as associated with any particular one of the ordinary mental

1 Because of the absence of details in regard to the method employed by Richardson, his work will not be considered in the following discussion.

2 *Die periodischen Geistesstörungen*, Jena, 1901.

3 *Amer. Journ. of Insan.*, April, 1902.

4 *Ibid.*, lx, p. 699, 1904; lxi, p. 299, 1904.

disorders." An approximate estimation of the urinary indican was made in each of Folin's cases. Examination of the data shows marked variations in the elimination of this substance in the different classes of mental disorders, in each class, and in the daily elimination of each individual. These facts, together with the comparatively slight difference from the excretion of normal persons, make clear the difficulty of proving an increase or decrease in the indican output as characteristic of any one class or phase of mental disease.

The most recent worker in this particular field, and the most positive advocate of the toxic influence of indoxyl in "melancholia" is Townsend. According to the results of his work,[1] sufferers from melancholia[2] (?) excrete, without exception, large amounts of indoxyl in the urine, which continues in pathological quantities until the clearing up of the psychosis. At the same time, they exhibit marked "symptoms and signs of toxemia" of intestinal origin, which also vanish simultaneously with the disappearance of the abnormal amount of indican. Townsend found no increase of indoxyl in other functional mental disorders. There seems, then, to be considerable evidence to show that there is a large excess above normal in the elimination of indoxyl sulphate in some of the non-organic insanities. On examining the evidence, however, no two observers are found in agreement as to the relation indoxyl bears to the mental disturbance, nor to the type of insanity of which it is characteristic, opinions on this point ranging from Richardson—constant and characteristic increase in all forms of psychosis—to Folin—constant and characteristic increase in none; Coriat finding always low figures in "kinetic" cases and melancholia of the involution period, and Townsend his highest in melancholia with agitation and great activity.

Considering this status of the question in regard to indoxyl elimination, and its relation to the functional insanities, it seemed worth while to endeavor to assist in clearing up the question by a series of quantitative determinations on a sufficient number of cases, carried over a long period and controlled by similar estimations on healthy individuals.

[1] *Journ. of Ment. Sci.*, li, No. 212, 1905.

[2] Probably several distinct types of depression including the melancholia of the involution period.

NEED OF QUANTITATIVE TESTS.

The necessity for making the determinations quantitative becomes evident from the consideration of several points apparently not appreciated by most previous workers.

1. It is fallacious to form positive judgments of excretory efficiency or abnormality from the merely qualitative estimation of any of the urinary constituents. That indican is no exception in this respect will be seen by a reference to Table V.

2. If there is a danger of error in judgment from qualitative methods of estimation in a single test, the danger is certainly augmented when a large number of such questionable judgments are collected and compared, and a rule or an apparent fact deduced therefrom.

3. The work of Folin, to which reference at greater length will be made subsequently, has shown that even in healthy "normal" subjects the variation in the amount of urine excreted from day to day even by the same individual under constant conditions of diet and exercise, is astonishingly great, and that it is even more marked in the case of the insane. It is clear that such variations will be maximal where little or no effort is made to have diet and exercise conform to a standard, or where, even with the will to do so, it is impossible, as in the case of disturbed patients.

The necessity for accurate controls in all investigation is so evident that the point hardly requires mention, and yet but one of the observers[1] to whose work reference has been made, devoted any attention to the control of his work by similar estimations on the urine of "normal" subjects.

CHOICE OF METHOD.

Of the methods employed by previous workers, only one—that of Folin—was at all adapted to the end in view, and even it, although accurate and in a way quantitative, is not entirely satisfactory, because it becomes necessary in using it to express values obtained according to a particular color scale, which precludes ready comparison with previous quantitative determinations of indican.

[1] Folin.

The Obermayer–Wang–Ellinger method is without objection, except from the point of time and intricacy. The method of Strauss, though perhaps not as accurate as Ellinger's method, is based on the same principles, admits of rapid estimation, gives results in milligrams, and is approximately accurate.

This method[1] was chosen, and to determine the extent of error comparisons were made at different times during the year's work with the Wang–Ellinger method. The indican in six 24-hour quantities was determined by both methods, with the following results:

Date.	Amount of Urine.	Ellinger's Method.	Strauss's Method.	Qualitative.
VII, 21, '05	600 c.c.	.0033 gm.	.0041 gm.	Blue,
" 19, '05	500 "	.0033 "	.0047 "	Dark blue,
" 26, '05	360 "	.0144 "	.00135 "	Intensely dark blue,
" 27, '05	650 "	.0086 "	.0073 "	Very dark blue,
X, 14, '05	1200 "	.0074 "	.007 "	Blue,
II, 21, '06	1100 "	.0245 "	.0022 "	Intense blue.

As funnels for the chloroform extraction, the Strauss funnel for the determination of lactic acid proved satisfactory. Obermayer's reagent, 20 per cent. lead acetate, and chloroform, were measured from burettes, and the method thus made more expeditious, without diminishing its accuracy. As tubes for comparison of the chloroform extracts, Purdy's graduated centrifuge tubes were found especially well adapted as the varying caliber and the graduations facilitated accurate comparison and a rapid estimate of the amount of chloroform used in dilution. In testing various strengths of Obermayer's

[1] Twenty c.c. of urine are precipitated with 5 c.c. of 20 per cent. lead acetate solution and filtered. Of the filtrate 10 c.c., corresponding to 8 c.c. of urine, are taken. To this 10 c.c. of Obermayer's reagent are added in a small separating funnel and in addition 5 c.c. of chloroform. The whole is thoroughly shaken, allowed to stand for 1 or 2 minutes, and again shaken. The chloroform is run off and a second extraction with 5 c.c. of fresh chloroform made. This is continued till the chloroform returns colorless. Of the united extracts, 2 c.c. are received in a tube of the same caliber as that holding the comparison fluid containing 1 mgm. of Kahlbaum's indigotin to the liter of chloroform. Dilution of this with chloroform is continued till the color tint in the tubes when held against a white background is identical and the amount of chloroform required noted. If the original extraction required 15 c.c. and the dilution 10 c.c. more and the excretion of urine for 24 hours was 860 c.c., then [illegible] = .00967 or 9.67 mgms. indigo from the day's urine.

reagent, it was found that if the quantity of ferric chloride exceeded two parts per thousand, there was danger of oxidation of any indigo formed into isatin or other colorless compound. Where the volume of urine was large, and the total amount of indoxyl not great so that the chloroform extract from 10 c.c. was only light blue, the use of the stronger reagent might so overoxidize as to give the impression that no indoxyl existed in the specimen. It was found, in fact, that exactly 2 grams of ferric chloride per liter of fuming hydrochloric acid was the optimum strength, and all the reagent used was carefully prepared according to this formula by dilution from a stronger solution.

In like manner, the chloroform comparison-solution of indigotin—one milligram to the liter—was made up from time to time from Kahlbaum's indigotin, a solution containing 25 milligrams per liter being made first. The stock solution was kept in a light-proof closet, and the comparison tube filled therefrom every second day.

The statement has recently been made that there frequently occur urines containing considerable amounts of indoxyl sulphate, the filtrates from which, after precipitation by lead acetate, are not oxidized to indigo by Obermayer's reagent, but may be so oxidized by the further addition of a dilute solution of potassium chlorate. The truth of this could not be confirmed during the present work, and it is thought that if such urines exist, they are of too infrequent occurrence to exert any influence on the validity of the results of this investigation. It is believed further, that any error caused by such would be equally great in determinations made by the Wang–Obermayer–Ellinger method, as the principles involved are the same.

WHAT MAY BE CONSIDERED NORMAL.

To reach an opinion based on personal experience, of what may be considered a "normal" amount of indoxyl to be passed daily by a person in average health, some eighty odd examinations were made of the urine of thirty people in different walks of life, who were in fair health and attending uninterruptedly to their work and not "nervous" or subject to "blues" or other depressed states. The average daily excretion as based upon this estimate is 5.9 milligrams, the extremes varying from practical absence to 32 milli-

grams in the 24 hours (Table I). (The latter figure was found in the urine of a hospital interne who at the time was suffering from a severe migraine or bilious attack, with intense headache, diarrhœa, and vomiting. It is the only instance included in the averages in which the individual did not feel perfectly well.) It seems then compatible with health, for a person to excrete daily over a considerable period anywhere from 5 to 10 milligrams of indoxyl while an occasional excretion of 15–20 milligrams may occur without causing any notable symptoms.

TABLE I SHOWING INDOXYL EXCRETION IN HEALTHY SUBJECTS.

Subject.	Date.	Volume of Urine. c.c.	Color of Urine.	Sp. Gr.	Indican. Grams.	Color of Chloroform Extract.
Dr. M., State Hospital	III, 7,'06	1060	Amber	—	.0018	Pale blue
	" 8, "	1010	"	—	.003	Light "
	" 9, "	1510	"	—	.0045	" "
	" 10, "	1250	"	1024	.0032	" "
Dr. R., State Hospital	III, 9,'06	1100	Amber	—	.0082	Dark blue
	" 11, "	1130	"	1029	.0056	Blue
	" 12, "	820	"	1028	.0077	Dark blue
	" 13, "	760	"	1031	.0066	" "
	" 14, "	870	"	1032	.0087	" "
Dr. B., State Hospital	III, 12,'06	900	Amber	1030	.0034	Light blue
	" 13, "	1050	"	1030	.0023	" "
	" 14, "	1100	"	1028	.0035	" "
	" 15, "	1080	"	1027	.0038	" "
Mrs. E., Perfect Health	XII, 22,'05	1300	Amber	1023	.0111	Blue
	" 27, "	1300	"	1028	.0123	Dark blue
	" 29, "	1250	Light amber	1025	.0117	" "
	" 30, "	1300	Amber	1023	.0001	Pale "
	" 31, "	1325	"	1022	.0158	Deep "
	I, 2,'06	1175	Light amber	1024	.0017	Pale "
	" 4, "	1150	Straw	1027	.0028	Light "
Dr. B., State Hospital	XII, 6, '05	1120	Amber	1029	.0077	Blue
	" 7, "	1210	"	1030	.0027	Pale blue
Dr. C., State Hospital	XII, 6,'05	1100	Amber	1025	.0028	Light blue
	" 7, "	1120	"	1031	.0014	Pale "
	" 8, "	1060	"	1031	.0051	Light "
	" 12, "	1550	"	1022	.0000	Colorless
	" 13, "	930	Dark amber	1029	.0089	Light blue
Dr. B., Institute Staff	I, 4,'06	500	Amber	1024	.0032	Blue
	" 10, "	900	"	1028	.0028	"
	" 11, "	750	"	1026	.0051	"
	" 12, "	890	"	1028	.005	Dark blue
Nurse No. I	IV, 26,'06	1010	Amber	1020	.016	Very dark blue
	" 27, "	1240	"	1022	.0078	Blue
	" 28, "	2010	Light amber	1014	.004	Light blue
Nurse No. II	IV, 27,'06	1420	Amber	1023	.0048	Light blue
	" 28, "	1020	"	1019	.019	Intense "
	" 29, "	1660	"	1018	.0062	Blue

TABLE I (CONTINUED.)

Subject.	Date.	Volume of Urine. c.c.	Color of Urine.	Sp. Gr.	Indican. Grams.	Color of Chloroform Extract.
Dr. R., Institute Staff	VI, 15,'05	1250	Amber	1018	.0043	Blue
	" 16, "	950	Dark amber	1024	.0196	Intense blue
Dr. R., Institute Staff	VI, 16,'05	750	Dark amber	1029	.014	Intense blue
	" 17, "	660	Dark amber	1030	.0105	" "
Mr. M., "Diener" in Laboratory	VI, 12 '05	1400	Amber	1025	.0113	Dark blue
	" 13, "	1200	"	1026	.0033	Light "
	" 15, "	1160	Orange	1026	.0119	Very dark blue
	" 16, "	1050	"	1032	.0078	Dark blue
Dr. C., Institute Staff	VI, 5,'05	1325	Amber	1024	.0082	Dark blue
	" 6, "	975	"	1027	.006	" "
	" 7, "	720	"	1030	.0054	" "
	" 8, "	1270	"	1025	.0019	Pale "
	" 9, "	1030	Pale amber	1026	.0008	Very pale blue
	" 10, "	680	Amber	1030	.0053	Dark blue
	" 11, "	1140	"	1028	.0057	Blue
	" 13, "	875	"	1028	.002	Light blue
	" 15, "	920	"	1028	.0057	Dark "
	" 16, "	1000	"	1031	.0034	Light "
	" 17, "	775	"	1028	.0029	" "
	" 19, "	1030	Pale amber	1026	.0038	" "
Dr. P., State Hospital Staff	XII, 5,'05	930	Dark amber	1033	.0064	Blue
	" 6, "	620	Dark amber	1034	.0031	Dark blue
	" 8, "	1050	Amber	1033	.0065	Blue
Dr. W., State Hospital Staff	XII, 7,'05	1410	Light amber	1034	.0089	Blue
	" 8, "	1210	Amber	1030	.0131	Very dark blue
	" 9, "	2010	"	1028	.032*	Intense blue
	" 13, "	1320	"	1029	.0086	Blue
	" 19, "	1540	"	1028	.002	Pale blue
Dr. N., State Hospital	XII, 6, '05	980	Amber	1024	.003	Light blue
	" 8, "	1200	"	1022	.0027	Pale "
	" 12, "	1150	"	1020	.0000	Colorless
Dr. S., State Hospital	XII, 8, '05	920	Amber	1029	.0146	Very dark blue
	" 6, "	1250	"	1030	.0093	Dark blue
House Staff St. Luke's Hospital						
Dr. Hu.	XII, 6, '05	950	Amber	1021	.0018	Pale blue
" He.	" " "	1100	Yellow	1025	.0062	Blue
" Ha.	" " "	2250	Pale straw	1007	.0014	Pale blue
" T.	" " "	1900	Light straw	1016	.0036	Light "
" Hus.	" " "	650	Amber	1022	.0017	Blue
" W.	" " "	1000	Light amber	1024	.0062	"
" B.	" " "	650	Amber	1025	.0045	Dark blue
" M.	" " "	1200	Light amber	1014	.0052	Blue
" C.	" " "	1350	Light amber	—	.011	Dark blue
" Ch.	" " "	1400	Light amber	1022	.0043	Light blue
(Boy) Arthur	" " "	2800	Pale straw	—	.0012	Very pale blue
Dr. L.	" " "	1000	Straw	1023	.0056	Blue
Mrs. ———	" 22, "	500	amber	1030	.00075	Pale blue

* Intense headache, vomiting, diarrhœa.

DETERMINATIONS ON AVERAGE WARD CASES.

For the purpose of orientation, a series of examinations on several different types of insanity was made. These comprised the cases whose histories are given by Dr. Kirby at the end of this paper (Series I).

In all, 213 examinations were made on 19 different female patients upon routine ward diet.[1] The indican was found to average low. In four markedly stuporous cases,—H. Loenecke, Hackett, Martin, and Josephs, the averages for one month of daily examinations were: 0.0026, 0.0025, 0.0026, 0.0024, and the extremes were from a bare trace to 0.0095 gram. Such results necessitate no elaborate charts to show the relations more minutely.

Of the other ward cases, the highest daily elimination was 19 milligrams in the case of E. T. In 6 instances of the 213, indican could be said to be practically absent, as the chloroform returned colorless.

During this period the patients received cathartics—compound cathartic pills—not oftener, as a rule, than once in two or three weeks, and an occasional enema.

The color reactions showed the range of shades noted by Coriat and Townsend. The tint of the chloroform after extraction varied from absence of color to a very dark blue shade. But the latter was almost always accompanied by a concentration of the urine; when not, the indican was indeed somewhat but not "enormously" increased.

There was nothing to indicate any constancy in the elimination of indican in any particular type or phase of insanity. It became desirable to investigate the question in acute cases likely to recover, and to begin the collection of urine as soon as possible after the admission of each case.

[1] The following is a sample of this diet—June 12, 1905—Breakfast: oatmeal, bread, butter, coffee. Dinner: soup, bread and beans, potatoes, pickles. Supper: bread and butter, tea, salt fish. June 13th.—Breakfast: corn-meal and milk, bread, butter, and coffee. Dinner: meat, potatoes, spinnach, bread and pudding. Supper: bread, butter, tea, rice, syrup. The patients are practically unrestricted as to quantity.

EXCRETION IN ACUTELY INSANE PATIENTS.

The attempt to carry out this plan resulted in a study of the urine of ten cases, seven of which were of a recoverable type. The study included the estimation of indican, and, in certain instances, that of phenol, skatol (Herter's adaptation of Ehrlich's dimethylamidobenzaldehyde reaction), and the combined sulphates as a whole. The study of phenol and skatol was soon discontinued, as the reactions were seldom intense enough to warrant any conclusions relative to unusual increase or change from normal. The sulphate ratio was so frequently low and so seldom high, that its study was early abandoned, though not before it was definitely shown that there would be little profit in continuing it.

Attention was concentrated on the indoxyl output, and on the variation in amount of urine excreted. No attempt to institute a special diet was made, first because of the impracticability of such an undertaking, with disturbed patients, unless recourse was had to forced feeding, and, second, because it seemed desirable to imitate, in so far as compatible with accuracy, the methods of previous observers, in order to give a better basis for comparison of results with theirs.

The most striking point brought out was the fact that in a large proportion of acutely insane patients of whatever type, but especially in cases of depression, the excretion of urine is diminished in the early stages, and that, as recovery progresses, the amount of urine voided commensurately increases. The regularity of this phenomenon will be seen by a glance at the accompanying table (II). These cases were consecutive admissions, entirely unselected, except in so far as was necessary to bring them within the class of acute insanities. The influence of such alterations in the amount of urine upon the apparent amount of indoxyl when estimated qualitatively will be evident on examining the charts, and notably Table V. As regards the actual indoxyl output, in not a single instance could it be called excessive in the light of what had been found in the normal cases.

TABLE II SHOWING VOLUME OF URINE AND INDOXYL EXCRETION IN TEN CASES OF INSANITY OF ACUTE, RECOVERABLE TYPE DURING THEIR EARLY STAY IN THE HOSPITAL.

Name and Diagnosis.	Date.	Volume of Urine. c.c.	Color of Urine.	Sp. Gr.	Indoxyl. Grams.	Color of Chloroform Extract.
Annie Brett* General Paralysis	III, 16,'06	810	Amber	1022	.006	Dark blue
	" 17, "	875	"	1023	.0044	Blue
	" 21"**	1050	"	1010	.0046	"
	" 23, "	425	"	1021	.0105	Intense blue
Non-Recoverable	IV, 19, "	2100	"	1012	.0047	Light blue
	" 20, "	1490	"	1013	.0054	" "
	" 21, "	1740	"	1013	.0074	Blue
	" 22, "	2030	"	1013	.005	Light blue
	" 23, "	1150	"	1018	.0014	Very pale blue
	" 24, "	750	"	1017	.002	Pale blue
Jennie Parker† Manic-depressive Insanity Kinetic	III, 9,'06	780	Muddy brown	—	.0219	Intense blue
	" 13, "	1450	Amber	—	.0118	Very dark blue
	" 14, "	610	"	—	.0007	Very pale blue
	" 17, "	600	"	1014	.0003	
	" 21, "	620	"	1012	Trace	
	IV, 19, "	1100	—	—	—	—
	" 20, "	1400	Amber	1020	.007	Blue
	" 21, "	2120	"	1016	.0015	Very pale blue
	" 22, "	1780	"	1022	.011	Dark blue
	" 23, "	1680	"	1021	.0016	Very pale blue
	" 24, "	1930	"	1020	.007	Blue
Lizzie Little†† Alcoholic psychosis Akinetic	II, 24,'06	400	Dark amber	1024	.002	Blue
	" 28, "	320	" "	1030	.0072	Intense blue
	III, 1, "	190	" "	—	.0016	Dark blue
	" 2, "	510	" "	—	.0006	Light "
	" 3, "	260	" "	1029	.0029	Dark "
	" 26, "	620	" "	1014	.0024	Blue
	" 27, "	520	" "	1014	.0037	"
	" 29, "	160	" "	1022	.001	"
	" 30, "	300	" "	1028	.0034	Dark blue
	IV, 19, "	1100	Amber	—	—	—
	" 20, "	1020	"	1018	.0033	Light blue
	" 21, "	250	"	1020	.0053	Intense "
	" 22, "	1510	"	1014	.0084	Blue
	" 23, "	380	"	1018	.0034	Dark blue
	" 24, "	1155	"	1017	.0065	Blue

*Admitted III, 14, '06. Sleep poor, appetite poor, bowels somewhat constipated.
IV, 24, '06. Sleep irregular appetite good, bowels rather free.

**Laxative administered.

†Admitted III, 6, '06. Tongue coated, bowels constipated, sordes on lips and teeth, depressed, apprehensive.
IV, 24, '06. Appetite good, bowels free.

†† Admitted II, 22, '06. Tongue heavily coated, bowels constipated, gastric contents normal.
IV, 24, '06. Appetite fair, sleep good, bowels free.

TABLE II (CONTINUED.)

Name and Diagnosis.	Date.	Volume of Urine. c.c.	Color of Urine.	Sp. Gr.	Indoxyl. Grams.	Color of Chloroform Extract.
Mary Mohr‡ Alcoholic Psychosis Kinetic.	II, 28, '06	450	Dark amber	1029	.006	Pale blue
	III, 1, "	475	Amber	—	.0005	" "
	" 2, "	420	Dark amber	—	.0002	Very pale blue
	" 3, "	510	Muddy amber	1029	.0014	Pale blue
	" 4, "	670	Orange	1024	.0021	Light "
	" 25, "	750	Amber	1022	.007	Dark blue
	" 26, "	890	"	1022	.011	Deep "
	" 27, "	500	"	1028	.005	Dark "
	" 29, "	820	"	1029	.0038	Blue
	" 30, "	900	"	1027	.009	Dark blue
	IV, 19, "	900	"			
	" 20, "	820	"	1020	.0033	Light "
	" 21, "	840	"	1013	.0033	" "
	" 22, "	1370	"	1010	.001	Pale "
	" 23, "	845	"	1012	.0031	Light "
	" 24, "	1365	"	1010	.0041	Blue
Mary McNulty‡‡ Dementia Præcox Akinetic	II, 16, '06	450	Dark amber	1027	.002	Blue
	" 21, "	850	Amber	1016	.0042	"
	" 22, "	440	Dark amber	1027	.0017	Light blue
Non-Recoverable	III, 23, "	700	Amber	—	.0017	" "
	" 24, "	525	Dark amber	1014	—	—
	" 26, "	425	Amber	1016	.0013	Light blue
	" 27, "	300	"	1030	.002	Blue
	" 28, "	580	"	1022	.0022	Light blue
	" 29, "	450	"	1024	.0012	" "
	" 30, "	1125	"	1025	.0015	" "
	IV, 19. "	600				
	" 20, "	400	"	1034	.0021	Blue
	" 21, "	410	"	1012	.0015	Light blue
	" 22, "	680	"	1018	.0017	Pale "
	" 23, "	355	"	1018	.0016	Light "
Lizzie Regan§ Dementia Præcox Akinetic	XII, 28, '06	300	Yellow	1037	.001	Light blue
	" 29, "	300	Amber	1024	.0031	" "
	" 30, "	1000	"	1018	.0056	Blue
	I, 1, "	350	"	1030	.002	"
Non-Recoverable	" 4, "	400	Clear amber	1011	.0012	Pale blue
	" 5, "	400	Amber	1027	.0045	Deep "
	" 31, "	1270	Pale amber	1012	.001	Pale "
	II, 1, "	1900	" "	1013	.005	Light "
	" 2, "	500	Amber	1019	.0045	Dark "
	" 3, "	1500	"	1022	.0095	Blue
	" 4, "	1010	"	1014	.005	"
	" 5, "	1450	"	1012	.0087	"

‡ Admitted, II, 28, '06. Tongue heavily coated, breath foul, bowels constipated.
III, 30, " Regular bowels,—fair appetite.
IV, 24, " Sleep good, appetite good, bowels free, marked improvement physically and mentally much the same.

‡‡Admitted II, 12, '06 Tongue coated, bowels constipated, appetite poor, sleeps well.
III, 30, " Sleeps well, bowels free, appetite good.
IV, 23, " Appetite good, bowels regular, sleep regular.

§ Physical improvement marked, mental improvement not great.

TABLE II (CONTINUED).

Name and Diagnosis.	Date.	Volume of Urine. c.c.	Color of Urine.	Sp. Gr.	Indoxyl. Grams.	Color of Chloroform Extract.
Straits	XII, 28,'06	350	Dark amber	1033	.0026	Dark blue
Allied to manic-	" 29, "	400	Yellow	1024	.0045	Deep "
depressive in-	" 30, "	300	Orange	1028	.0024	Dark "
sanity. Akinetic	" 31, "	400	Yellow	1128	.005	Deep "
with agitation	I, 1, "	400	Amber	1024	.0042	" "
	II, 1, "	650	"	1010	—	
	" 2, "	660	"	1010	.0033	Blue
	" 3, "	520	"	1014	.0039	"
	" 4,	700	"	1010	.001	Pale blue
	" 5, "	1000	"	1012	.0015	" "
	IV, 19 "	1120				
	20, "	880	"	1015	.0027	Very light blue
	" 21, "	410	"	1030	.0092	Intense blue
	" 22, "	1225	"	1020	.0092	Dark blue
	" 23, "	230	"	1036	.0023	" "
	" 24, "	830	"	1013	.0078	" "
Barbara Schmidt§§	I, 4,'06	900	Amber	1034	.0126	Dark blue
Infective-ex-	" 6, "	1000	"	1033	.015	Deep "
haustive psycho-	" 7, "	400	"	1032	.005	Dark "
sis Akinetic	" 9, "	400	"	1030	.0037	" "
	" 12, "	360	Dark amber	1031	.0038	" "
	II, 29, "	800	" "	1017	.003	Light "
	" 30, "	2010	Amber	1010	.004	Blue
	" 31, "	825	"	1014	.005	Dark blue
	III, 1, "	1180	"	1010	Trace	—
	" 2, "	900	"	1011	.0067	Dark blue
	IV, 19, "	920	Dark amber			
	" 20, "	810	" "	1010	.007	Dark blue
	" 21, "	390	" "	1014	.0046	" "
	" 23, "	410	" "	1020	.0077	Intense "
	" 24. "	490	"	1010	.0045	Dark "
McNaner***	I, 13, 06	430	Amber	1025		
	" 14, "	510	"	1023		
Manic-Depressive Insanity	" 15, "	370	"	1027	.001	Light blue
Akinetic	" 16, "	450	"	1011	Trace	
	" 30, "	1100	"	1015	.003	Pale blue
	" 31, "	1340	"	1008	.0016	" "
	II, 1, "	1030	"	1015	.0025	" "
	" 2, "	1450	"	1011	.002	" "
	" 3, "	2080	"	1012	.0026	Very pale blue
	" 14, "	1670	"	1010	.008	Blue
	" 15, "	1130	"	1019	.0056	"
	" 16, "	1800	Pale amber	1011	.0044	Light blue
	" 17, "	2000	" "	1015	.0075	" "

§§ Admitted, I, 2, '06. No appetite, sleep poor.
II, 2, " Appetite good.
IV, 24, " Appetite fair, bowels free, sleep good, no laxatives, mental improvement not marked.

*** Admitted, I, 12, '06. Gradual mental and physical improvement.

TABLE II (CONTINUED).

Name and Diagnosis.	Date.	Volume of Urine. c.c.	Color of Urine.	Sp. Gr.	Indoxyl. Grams.	Color of Chloroform Extact.
Martin	V, 4, 05	550	Orange	1018	.0007	Pale blue
	" 5, "	320	"	1020	.0014	Light "
Alcoholic Hallucinosis Tendency to Stupor	" 6, "	250	Dark orange	1022	.003	Dark "
	" 7, "	500	Orange turbid	1021	.0057	" "
	" 8, "	280	" "	1014	.0036	" "
	" 19, "	900	Amber	1012	.0022	Light "
	" 21, "	550	"	1015	.0019	" "
	" 22, "	650	"	1016	.0026	Blue
	" 24, "	300	Clear amber	1027	.0009	Light blue
	" 25, "	850	Amber		.0029	" "
	" 26, "	520	"	1018	.0014	" "
	VI, 1, "	800	Light amber	1018	.0075	Dark blue
	" 2, "	600	" "	1013	.0024	Blue
	" 3, "	925	Amber	1018	.0019	Pale blue
	" 4, "	990	"	1016	.0037	Blue
	" 5, "	550	"	1018	.0055	"
	" 6, "	875	Light amber	1017	.0005	Pale blue

EFFECT OF DIET.

A diet test was made to determine the accuracy of Coriat's contention that variation in indican excretion in pathological mental conditions is "entirely independent of . . . diet . . . and simply an at present unexplainable, concomitant symptom of the akinetic or hyperkinetic state."

Two diets were used, those employed by Folin in his extensive metabolism work.[1] One is a liquid, full-nitrogen "standard diet." The ingredients are milk, cream, eggs, water, sugar, salt, and Horlick's malted milk, mixed in definite proportions so that the content varies practically not at all from day to day. The second diet is a non-nitrogenous one, of pure arrowroot and cream.

Seven patients were selected,—three active "kinetic" cases, four depressed, "akinetic,"—the latter very marked of their type. All cathartics and enemata were stopped. The urine was examined for a few days while the patients were on the routine ward diet. During a second period they received the standard liquid diet and finally the "kinetic" cases went back to routine diet, whereas the "akinetic" cases were given the arrowroot and cream.

The accompanying table (III) summarizes the results. As a

[1] *Amer. Journ. of Physiol.*, xiii, pp. 45 and 66, 1905.

rule the standard liquid diet, which is rich in proteid, slightly raised the indican elimination, though there are exceptions to this. The starch cream diet caused a practical disappearance of indican in every instance at the end of the first 48 hours succeeding its introduction. With the exception of the third period, the color reactions varied from a very deep blue—almost black—to a pale blue. The majority were dark blue, and a very few were practically colorless. There were no constant relationships in total amount of indican excreted, and similar variations were found in all patients of both types.

TABLE III.

ACTIVE HYPERKINETIC CASES.

Rabner.*

Diet.	Date.	Volume of Urine. c.c.	Sp. Gr.	Color of Chloroform Extract.	Amount of Indican. Grams.
Routine	VII, 16, '06	1000	1020	Very dark blue	.0125
	" 17, "	1550	1017	" " "	.017
	" 18, "	950	1017	Dark blue	.0077
	" 19, "	775	1019	" blue	.0063
	Averages	1068			.0109
Standard Diet (Folin)	VII, 20, '06	675	1018	Dark blue	.0069
	" 21, "	675	1026	Very dark blue	.0088
	" 22, "	725	1027	Blue	.005
	" 23, "	690	1028	"	.0056
	" 24, "	840	1025	Dark blue	.0078
	" 25, "	750	1025	Very dark blue	.0103
	" 26, "	650	1026	Blue	.004
	Averages	715			.0069
Routine Diet	VII, 27 '06	580	1022	Blue	.0032
	" 29, "	1125	1013	Almost colorless	Trace
	" 30, "	1980	1014	Very light blue	.001
	" 31, "	1950	1015	Pale blue	.005
	Averages	1409			.001

Emerick.

Diet.	Date.	Volume of Urine. c.c.	Sp. Gr.	Color of Chloroform Extract.	Amount of Indican. Grams.
Routine	VII, 16, '06	760	1026	Blue	.0033
	" 17, "	600	1026	"	.003
	" 18, "	550	1026	"	.0027
	" 19, "	500	1025	Dark blue	.0047
	Averages	602			.0034
Standard Diet (Folin)	VII, 20, '06	400	1027	Dark blue	.0045
	" 21, "	500	1032	Very dark blue	.0062
	" 22, "	675	1033	Blue	.0038
	" 23, "	500	1035	Dark blue	.004
	" 24, "	475	1031	Blue	.0032
	" 25, "	625	1035	Intense blue	.0105
	" 26, "	360	1031	Very intense blue	.0135
	Averages	505			.0065
Routine Diet	VII, 27, '06	650	1024	Dark blue	.0073
	" 29, "	325	1023	Blue	.0022
	" 30, "	625	1020	Very light blue	.0008
	" 31, "	650	1022	Blue	.0047
	Averages	562			.0038

* During this patient's period of depression (see clinical notes) no increase in the amount of indican excreted could be noted. There was a slight decrease in the amount of urine excreted, the average daily quantity for 17 consecutive days, Oct. 19, to Nov. 18, being 802 c.c. The indican for the same period averaged 4.2 mgms.

TABLE III (CONTINUED).

M'Gauley.

Diet.	Date.	Volume of Urine, c.c.	Sp. Gr.	Color of Chloroform Extract.	Amount of Indican. Grams.
Standard Diet (Folin)	VII, 20, '06	600	1030	Blue	.0041
	" 21, "	175	1028	Dark blue	.0014
	" 22, "	430	1030	Blue	.0028
	" 23, "	660	1031	"	.0049
	" 24, "	900	1026	"	.0061
	" 25, "	950	1022	Very dark blue	.0142
	" 26, "				
	Averages	619			.0056
Routine	VII, 27, '06	525	1031	Blue	.0029
	" 29, "	550	1023	Very pale blue	.0005
	" 30, "	1150	1023	" " "	.0014
	" 31, "	1385	1019	" " "	.001
	Averages	903			.0014

DEPRESSED AKINETIC CASES.

Gray.

Diet.	Date.	Volume of Urine, c.c.	Sp. Gr.	Color of Chloroform Extract.	Amount of Indican. Grams.
Routine Diet	VIII, 6 '06	1450	1013	Very light blue	.0018
	" 7, "	260	1023	" " "	.0006
	" 8, "	260	1028	Very dark blue	.0042
	" 9, "	550	1020	" " "	.0123
	" 10, "	880	1013	Blue	.0044
	" 11, "	675	1016	Dark blue	.0076
	" 12, "	425	1020	Very dark blue	.008
	" 13, "	325	1027	" " "	.0067
	" 14, "	175	1030	Intense blue	.004
	" 15, "	700	1013	Very light blue	.0005
	Averages	570			.005
Standard Diet	VIII, 16, '06	550	1020	Dark blue	.0061
	" 17, "	825	1015	Light "	.002
	" 18, "	900	1020	Very light blue	.0011
	" 19, "	1100	1013	" " "	.0014
	" 20, "	1100	1020	" " "	.0014
	" 21, "	875	1010	" " "	.002
	Averages	891			.0023
Starch-Cream Diet	VIII, 27, '06	1950	1008	Almost colorless	Trace
	" 28, "	875	1006	Colorless	
	" 29, "	1425	1006	"	
	Averages	1411			

Pomeroy.

Diet.	Date.	Volume of Urine, c.c.	Sp. Gr.	Color of Chloroform Extract.	Amount of Indican. Grams.
Routine	VIII, 9, '06	860	1017	Very light blue	.0026
	" 10, "	800	1022	Blue	.006
	" 11, "	775	1021	"	.0043
	" 12, "	775	1028	Dark blue	.0073
	" 13, "	900	1025	Light "	.0031
	" 14, "	860	1024	Blue	.0054
	" 15, "	1430	1016	Colorless	Practically absent
	Averages	914			.0041
Standard Diet	VIII, 16, '06	900	1027	Intense blue	.0225
	" 17, "	950	1022	Very dark blue	.0142
	" 18, "	900	1026	Intense blue	.0219
	" 19, "	295	1020	Light "	.0035
	" 20, "	900	1019	Blue	.0067
	" 21, "	910	1025	Dark blue	.0125
	Averages	809			.0135
Starch-Cream Diet	VIII, 27, '06	1000	1017	Very light blue	.005
	" 28, "	1200	1015	Colorless	—
	" 29, "	975	1009	"	—
	Averages	1058	—	—	—

TABLE III (CONTINUED).

Friedman.

Diet.	Date.	Volume of Urine. c.c.	Sp. Gr.	Color of Chloroform Extract.	Amount of Indican. Grams.
Routine	VIII, 6, '06	—	—	—	—
	" 7, "	—	—	—	—
	" 8, "	—	—	—	—
	" 9, "	875	1015	Very pale blue	.001
	" 10, "	950	1016	" " "	.0012
	" 11, "	1125	1015	" " "	.0019
	" 12, "	745	1016	Dark blue	.0065
	" 13, "	500	1022	Intense "	.0103
	" 14, "	1500	1014	Very pale blue	.0026
	" 15, "	1375	1010	Practically colorless	
	Averages	1010			.0033
Standard	VIII, 16, '06	675	1022	Intense blue	.0127
	" 17, "	850	1015	Blue	.0053
	" 18, "	975	1020	Dark blue	.0097
	" 19, "	1650	1015	Blue	.012
	" 20, "	890	1020	Dark blue	.0078
	" 21, "	1950	1012	Very light blue	.0031
	Averages	1165			.0084
Starch-Cream Diet	VIII, 27, 06				
	" 28, "				
	" 29, "				

Donohue.

Diet.	Date.	Volume of Urine. c.c.	Sp. Gr.	Color of Chloroform Extract.	Amount of Indican. Grams.
Routine	VIII, 6, '06				
	" 7, "	125	1027	Intense blue	.0043
	" 8, "	1500	1019	Dark "	.0106
	" 9, "	525	1018	" "	.0048
	" 10, "	1425	1013	Very pale blue	.001
	" 11, "	1675	1015	Almost colorless	Trace
	" 12, "	1375	1010	" "	Trace
	" 13, "	1175	1016	Very light blue	.0022
	" 14, "	1025	1013	" " "	.001
	" 15, "	2125	1010	" " "	.0027
	Averages	1216			.0029
Standard	VIII, 16, '06				
	" 17, "				
	" 18, "				
	" 19, "				
	" 20, "				
	" 21, "				
Starch-Cream Diet	VIII, 27, '06	950	1023	Practically colorless	—
					—
	" 28, "	950	1013	"	Trace
	" 29, "	1775	1014	"	—
	Averages	1225	—	—	—

EFFECT OF CONSTIPATION.

The question of the effect of constipation and catharsis upon the output of indoxyl in the urine has been much discussed. There is no doubt that in most cases of simple constipation—fecal accumulation in the rectum—very little influence is exerted. It is only when there is a stasis of the bowel contents in the small intestine and colon, with the production and retention there

of absorbable putrefactive products,—*i.e.*, indol and skatol,—that large amounts of indoxyl are to be found in the urine.[1] Conversely, cathartics which act almost entirely on the lower bowel will naturally have less influence in diminishing urinary indican than the combination of calomel with a saline which acts throughout the intestine and is strongly antiseptic as well. The present work substantiated on several occasions the fact that if the intestinal contents are kept in lively movement by the frequent administration of cathartics of any sort the output of indoxyl over a given period will be materially lessened, but it could not be shown that the occasional exhibition of a cathartic exerted any appreciable influence. In this connection it is interesting to note that indol itself acts as an efficient cathartic, large doses given by mouth almost always inciting diarrhœa, and that in this way it tends to prevent its own absorption. In considering the question of unusual amounts of indoxyl in the urine it should not be forgotten that this alone does not necessarily indicate increased putrefaction and production of indol in the bowel. A peculiar tendency to ready absorption or increased opportunity for absorption by stasis may give the same urinary appearances as increased production where these factors are not present. A failure to recognise this is no doubt responsible for many conflicting statements in regard to indol and indoxyl production.

As none of the insane patients studied in either the first or second series of cases exceeded the "normal cases" in the amount of indoxyl excreted, it appears clear that it cannot be demonstrated by trustworthy methods in acutely insane or chronic patients as found in hospital, that the output of indican is either constant or excessive, and it is evident that the effects of diet, constipation, or catharsis in diminishing or increasing the elimination of indoxyl in the urine of the insane are the same in kind and degree as in normal individuals.

EFFECT OF ADMINISTRATION OF INDOL.

Can indol production and absorption in the intestine be,

[1] Herter has recently shown that imperfect catharsis—the sweeping of large amounts of only partially digested food from the stomach and small intestine into the colon without moving it further—may be the cause of a greatly increased putrefaction and consequent production of indol.

nevertheless, a factor in determining the onset of a psychosis? In support of this, we have the experience of those who have examined their urine during attacks of mild depression ("blues") and found "a marked reaction" for indican. They took some calomel, the indoxyl disappeared, the world once more looked bright, and it seemed as though the indoxyl must have been at least one factor in causing the depression.

Herter[1] fed indol to rabbits, dogs, monkeys, and men. In some instances he observed depression, or other well-marked nervous manifestations. But certain animals, and men were markedly susceptible to its influence, 0.1 gram affecting them more than 1 gram did others.

Given a person prone to overindulgence in animal food, suffering from chronic gastro-intestinal catarrh, with impaired renal function, and a weak nervous system susceptible to the influence of indol, especially, if living more or less indoors under conditions of sub-oxidation, and it appears *a priori* that he would be not unlikely to develop a psychosis, most probably of a depressed type.

TABLE IV. SHOWING THE EFFECT OF ADMINISTRATION OF INDOL.

Case 1. Annie Diamond.*

Date.	Volume of Urine. c.c.	Color of Urine.	Sp. Gr.	Idoxyl. Grams-	Color of Chloroform Extract.	Indol Administration.
II, 14, '06	1210	Amber	1019	0.0025	Pale blue	
" 16, "	1200	"	1020	0 0048	Light "	
" 19, "	800	"	1024	0 0036	Blue	
" 21, "	1100	"	1020	0 0220	Intense blue	0 097 gm.
" 22, "	850	"	1018	0 0160	" "	

Case 2. Annie McHauer.

Date.	Volume of Urine. c.c.	Color of Urine.	Sp. Gr.	Idoxyl. Grams-	Color of Chloroform Extract.	Indol Administration.
II, 14, '06	1610	Amber	1018	0 0080	Blue	
" 15, "	1130	"	1019	0 0056	"	
" 16, "	1800	"	1011	0.0044	Pale blue	
" 17, "	2000	"	1015	0 0075	" "	
" 20, "	1840	"	1010	0.0259	Deep "	0 097 gm.
" 21, " †	1250	"	1013	0.0286	Intense "	
" 22, "	2200	"		0 0173	Dark "	0 065 gm.
" 23, "	840	"	1012	0 0031	Light "	
" 25, "	1010	Orange	1017	0 0031	" "	

* This patient was ready for discharge as cured; she was bright, up and about the wards working. She denied all subjective symptoms, had no diarrhœa and showed nothing beyond a dilatation of the pupils and moderate pallor.

† On this day the patient for the first time since admission spoke freely and voluntarily to the resident physician complaining of diarrhœa.

[1] *N. Y. Med. Journ.*, lxviii, 1898.

TABLE IV (CONTINUED).

Case 3. Josephine Callahan.‡

Date.	Volume of Urine. c.c.	Color of Urine.	Sp. Gr.	Indoxyl. Grams.	Color of Chloroform Extract.	Indol Administration.
III, 5, '06	1280	Amber	1015	0.0008	Pale blue	
" 6, "	1210	"		0.0039	Light "	0.032 gm.
" 7, "	1040	"		0.0038	" "	
" 8, "	960	"		0.0028	" "	
" 9, "	1070	"		0.0007	Pale blue	
" 10, "	950	"		0.0046	Blue	0.032 gm.
" 11, "	1050	"	1016	0.0081	Dark blue	0.032 "
" 12, "	700	"	1013	0.0068	Very dark blue	0.065 "
" 13, "	980	"	1018	0.0006	" pale "	0.065 "
" 14, "	1160	"	1014	0.0087	Blue	0.065 "
" 16, "	500	"	1017	0.0014	Pale blue	0.065 "
" 17, "	975	"	1020	0.0048	Blue	0.065 "
" 19, "	1050	"	1014	0.0080	"	0.065 "
" 20, "	525	"	1024	0.0060	Dark blue	0.065 "
" 21, "	1290	"	1019	0.0129	" "	0.065 "
" 22, "	1120	"	1026	0.0126	Deep "	0.065 "
" 23, "	570	"	1026	0.0060	" "	
" 25, "	1045	"	1015	0.0070	Blue	
" 26, "	1525	"	1010	0.0200	Intense blue	0.097 gm.
" 27, "	1300	"	1012	0.0032	Light "	0.097 "
" 29, "	1050	"	1026	0.0130	Deep "	0.097 "
" 30, "	800	"	1020	0.0090	" "	0.097 "

Case 4. Behsmann.**

Date.	Volume of Urine. c.c.	Color of Urine.	Sp. Gr.	Indoxyl. Grams.	Color of Chloroform Extract.	Indol Administration.
III, 5, '06	1400	Amber	1015	0.0031	Light blue	
" 6, "	670	"		0.0008	Very pale blue	0.032 gm.
" 7, "	1070	"		0.0040	Light blue	0.032 "
" 8, "	1090	"		0.0025	" "	
" 9, "	960	"		0.0025	" "	
" 10, "	330	"				
" 11, "	1450	"		0.0092	Dark blue	0.065 gm.
" 12, "	730	"		0.0084	Very dark blue	0.065 "
" 13, "	620	"	1010	0.0062	" " "	0.065 "
" 14, "	765	"	1010	0.0086	" " "	0.065 "
" 16, "	1575	"	1015	0.0149	Dark blue	0.065 "
" 17, "	1110	"	1012	0.0044	Light "	0.065 "
" 18, "	1535	"	1012	0.0153	Very dark blue	0.097 "
" 20, "	950	"	1020	0.0035	Blue	0.097 "
" 21, "	790	"	1011	0.0044	"	0.097 "
" 22, "	105	"	1030	0.0015	Dark blue	0.097 "

‡ The only noteworthy symptom in this experiment was severe diarrhœa.

** Moderate diarrhœa was produced; the patient finally refused to urinate or to take the capsules in which the indol was administered. Otherwise, no symptoms.

An endeavor to throw some light on this phase of the question was made by giving indol to certain recovered and partially recovered patients. The only constant effect noted (Table IV) was the establishment of a diarrhœa in all but one of the cases to which the indol was administered. Other results, except that of a notable increase of indoxyl sulphate in the urine, were of an ambiguous or entirely negative character. It is true that the dosages of indol were not large, but, on the other hand,

they were exhibited to patients of inferior and unstable type, among them those showing marked susceptibility to alcohol, and continued in some instances for several days, and it is noteworthy that practically no mental change of even transient nature occurred which could be attributed to the indol.

TABLE V SHOWING THE INFLUENCE OF CONCENTRATION OF URINE AND ACTUAL INCREASE OF INDICAN UPON THE COLOR REACTION.

Subject.	Date.	Volume of Urine. c.c.	Sp. Gr.	Color of Chloroform Extract.*	Indican Grams.
Hackett †	VI, 2, '06‡	100	1025	Intense blue	0.0021
	" 3, "	200	1025	" "	0.0035
	" 4, "	300	1025	" "	0.0047
	" 5, "	375	1025	" "	0.0068
	" 7, "	200	1025	Very dark blue	0.0026
Brodel	V, 24, '06	1750	1013	Light blue	0.0044
	" 26, "	1600	1010	" "	0.0045
	" 27, "	1150	1012	Dark "	0.0078
	VI, 5, "	1275	1019	Blue	0.0047
	" 13, "	1570	1013	Pale blue	0.0020
Rabner	VII, 16, '06	1000	1020	Very dark blue	0.0125
	" 17, "	1550	1017	" " "	0.0170
	" 18, "	950	1017	" "	0.0077
	" 19, "	775	1019	" "	0.0063
	" 27, "	750	1025	" " "	0.0103
Healthy persons	VI, 15, '06	875	1028	Light blue	0.0019
	" 16, "	950‡	1024	Intense "	0.0196
	" 16, "	750	1029	" "	0.0140
	" 15, "	1160	1026	Very dark blue	0.0119
	" 15, "	1250	1018	Blue	0.0043

* The color noted was that obtained as a result of the first extraction with 5 c.c. of chloroform.

† This patient never while under observation (40 days) excreted above 410 c.c. of urine in 24 hours.

‡ Note that the reaction was intense blue where there was only 100 c.c. of urine and 2.21 mgms. of indoxyl, as well as where there was 950 c.c. of urine and 19.6 mgms. of indoxyl; and that in the first test under Hackett and in the first normal case the amounts of indoxyl were practically equal but the color reactions very different.

Throughout all this work, the urine was collected in a divided vessel, so that there was never any admixture of feces.

DISCUSSION AND CONCLUSION.

So much of the results of this work is of an entirely negative nature, that it is necessary to attempt to account for the positive and definite findings of others.

The work of Pilcz is not extensive, and, like that of Coriat and Townsend, is based entirely upon qualitative determinations by the method of Jaffe. This test, once a standard one, is inade-

quate even for routine urine examination, and quite out of place in ostensibly scientific work. Unless performed with great care the reaction is worthless, even as an approximate indicator, and when all essentials of technic are complied with, the result may be quite *misleading, unless the total excretion of urine is noted*, and the strength of the reaction obtained considered in the light of this.

Coriat, Townsend, and Pilcz did not note the amount of urine passed in twenty-four hours, and did not make their tests on mixtures of the whole daily quantity but on single voidings, and, as has been noted, no attempt was made to control any of their observations by similar tests made on the urine of healthy, so-called "normal," subjects.

The extent and importance of the variations in amount of urine excreted from day to day even in healthy individuals has recently been brought to particular notice by Folin. He says [1]:

> "The volume of urine eliminated depends directly upon the amount of water consumed. But the variations in the volume are of considerable interest. Such changes are at times exceedingly pronounced in the same individual and different persons will on the same diet, habitually eliminate very different amounts of water with the urine. Changes of this kind cannot be accounted for by changes in weight and do not correspond to changes in atmospheric condition.
>
> "For example, on comparing the first periods of Tables II, III and X (analysis of the urine of three normal persons,) we find that on exactly the same amount of water consumed, the volumes of urine eliminated during the three days are 2555 c.c., 3770 c.c., and 5500 c.c. respectively, yet the loss of body weight is in each case about 300 grms., and the first two cases represent the same dates, thereby excluding the influence of the atmospheric conditions. Similarly, in Table IV, (*first period*,) we find the volume of urine varying from 780 c.c. to 2300 c.c. without change of diet and with but a slight loss of body weight. And in Table II (second period), we find the volume of urine varying from 385 c.c. to 1880 c.c."

The tables here shown and those of other workers confirm these facts. It is to be noted that the variations are not alone between different individuals, though these are most marked, but that the daily elimination by the same individual may show in the course of a few days large and at present unaccountable differences.

[1] *Amer. Journ. of Physiol.*, xiii, p. 100, 1905.

The daily production of urine varies greatly then even under absolutely uniform conditions of temperature, diet, atmosphere, etc. How much greater will be this variation in the case of insane patients of whom many and notably the "akinetic" ones are at times with difficulty made to drink. This is no doubt a factor which exerts marked influence in diminishing the urinary output in the early stages of insanities.

Townsend called attention to the fact that in many of his "melancholia" cases the sweat excretion was unusually great, the patients being continually bathed in moisture. This is a matter of not infrequent occurrence in such types. That this vicarious discharge of fluid may aid in concentrating the urine and thus increase the intensity of the indican reaction is evident and was well shown by a case at this hospital a record of whose urine and indican excretion are given in Table V (Hackett.)

It is evident that these several factors affecting the volume of urine passed might very well lead to error in estimating indoxyl elimination by test-tube methods alone, especially when the latter were faulty ones, and the tests made upon isolated specimens of single voidings, rather than upon samples from the mixed 24 hours' amount.

This work was undertaken with the aim of comparing and reconciling conflicting statements in regard to indoxyl excretion in the insane. There are certain difficulties inherent in such an investigation,—the peculiar class of patients,—the indefinite, loose, and inconstant terminology of mental disease,—and the variations in technic of previous workers. An attempt has been made, however, to show,—

1. That in spite of differences in nomenclature, it is clear that opinions on the question are widely variant.

2. That these divergent views may be put in two main groups,—(*a*) That of those who hold that in certain types of insanity indican elimination follows a characteristic and constant type. (*b*) That of those who believe that there is nothing constant or characteristic about it.

3. That those holding the former view employed methods of investigation of questionable accuracy,—that they neglected to control the work done,—and that, by reason of the methods

employed, they were led to attach exaggerated importance to differences in reaction which in reality denoted only slight or no difference in actual total excretion, and that in addition, no two of them were in agreement as to the type of insanity of which great or small amounts of indoxyl were characteristic.

4. That Herter and Folin,[1] who found in the classes of cases investigated nothing characteristic or constant,—employed approved quantitative methods of determination, and controlled their results by similar examinations of the urine of healthy subjects.

Finally, the result of the present investigation has gone to show that indol is not a factor of importance in determining the onset of depressive or other psychoses, and, although occasionally found somewhat increased at the beginning of such a psychosis, it is not constantly and seldom excessively increased, returns to normal long before there is any improvement in the patient's mental condition and bears no constant relation causative or other to any particular form of insanity.

The writer wishes to express sincere thanks to Dr. Adolf Meyer and Dr. Frank Wood for their many valuable suggestions and criticisms; also to Drs. Kirby and Holmes of the Institute for the preparation of the case-histories.

SUMMARIES OF CASE HISTORIES OF PATIENTS.

Cases 1–19 First Series.

1. E. T., age 39. Diagnosis: Depressive hallucinosis, tending to stupor. Duration, 3 months.

Under observation a condition of partial stupor has existed; she is without spontaneity in speech or action, but her movements are not retarded and she is free from rigidity or catalepsy. As a rule her face is stolid, and she gazes in a preoccupied way.

2. M. K., age 56. Diagnosis: Involution depression with a hypochondriacal trend and a well developed hysterical symptom-complex Duration, 4 months.

During the period of observation the patient was quiet, dejected, and inactive. Orientation good; no amnesias determined. General pain anæsthesia, absence of corneal and pharyngeal reflexes.

3. S. H., age 39. Diagnosis: Constitutional inferiority, with apathetic deterioration. Slow development since puberty.

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[1] Folin investigated almost all types of mental disease.

During the period of observation, the condition was one of profound apathy; she sat gazing all day, tending to assume a fixed position, never moving unless prompted, and not responding except in occasional monosyllables. Some aversion and resistance, but no rigidity or catalepsy.

4. M. B., age 46. Diagnosis: General paralysis, tabetic form, onset with hysterical symptoms. Duration 3 months. Husband syphilitic.

During the period when the urine was collected, all initiative was abolished; she displayed no interest, never spoke spontaneously or moved voluntarily from her chair. Absent knee jerks. Argyl-Robertson pupils, general diminution in pain sensibility; speech defect. Cerebrospinal fluid showed marked lymphocytosis.

5. H. L., age 20. Diagnosis: Dementia præcox. Duration, 3 months.

During the time when the urine was collected, she appeared dejected and preoccupied, spontaneous activity was much diminished; she sat gazing down, turned her face away when questioned, responded very little. In bed she wished to keep her head covered; she moved very seldom, and showed slight resistance in her arms in harmony with a general averse attitude.

6. C. H., age 21. Diagnosis: Dementia præcox—Duration 1½ years. The patient has been in a pseudo-stuporous condition for 6 months. She is profoundly apathetic and inactive. She sits all day in a constrained attitude, and never moves of her own accord. There is slight stiffness in the limbs, and a tendency to catalepsy.

7. C. B., age 40. Diagnosis: Manic-depressive insanity; two previous attacks; the present a manic attack of two months duration.

During the period of observation the patient was in a state of overactivity and mischievous behavior. With this motor excitement she kept up a steady stream of talk, which showed flights of ideas, or merely a reminiscent drifting from one topic to another.

8. M. M., age 47. Diagnosis: Alcoholic hallucinosis with delirious admixtures; tendency to stupor; 3 previous attacks of delirium tremens.

During the first week that the urine was collected, the patient was stuporous most of the time. There was a high degree of general rigidity; hallucinations at this time were abundant.

During the second week she began to improve, the hallucinations subsided, and the fear disappeared; she became bright and cheerful, and during the last three weeks she seemed to be in her normal condition.

9. Ida Joseph:

Admitted May 10, 1905. Age 28. Constitutional inferiority with recurrent outbreaks of impulsive acts and fits of uncontrollable temper. Improvement and discharge in about two months.

10. Mary Cavanagh:

Admitted May 18, 1905. Age 55. Manic-depressive insanity. 7 previous attacks with recovery.

11. Louise Forshee:

Admitted May 18, 1905. Age 30. Infective exhaustive psychosis of

sudden onset during lactation. Improved, and discharged in 7 months

12. CATHERINE LEDDY:

Admitted May 18, 1905. Age 49. Depressive hallucinosis occurring at the involution period. Subsidence of hallucinations in about 6 months' time, but persistence of an indifferent, lackadaisacal, ambitionless mood. Still in the hospital at the end of 11 months.

13. ANNA WHISKER:

Admitted May 20, 1905. Age 43. Recurrent paranoiac trend. Two previous attacks with improvement; some excess in alcohol. Still in the hospital at the end of 11 months.

14. CATHERINE PORFENOFF:

Admitted May 20, 1905. Age 50 (?); slight paranoiac wave after prolonged over-work and worry. Recovery and discharge in two months.

15. LIZZIE MELVILLE:

Admitted May 25, 1905. Age 21. Dementia præcox in an inferior individual manifesting itself during pregnancy and after a rheumatic attack—gradual decline. Still in the hospital after a period of 10 months.

16. EMILY CLINCH:

Admitted June 7, 1905. Age 33. Depression not sufficiently distinguished. Ill-defined hallucinations; gradual improvement. Still in the hospital at the end of 10 months.

17. ALBERTINA HUBBEL:

Admitted June 1, 1905. Age 39. Paranoiac condition of gradual development and six years duration. No defect. Still in the hospital after a period of 10 months.

18. B. P., age 23. Diagnosis: Dementia præcox. Duration 2 years.

The patient has been in a hypokinetic state with mutism for over a year.

During the period of the observation, there was no change in her condition. Considerable muscular tension is encountered in the limbs. She never changes her fixed position except to eat, and about this she is sloven and untidy.

19. S. F., age 19. Diagnosis: Dementia præcox (constitutional inferiority, followed by hebephrenic deterioration.) Duration four years.

In the hospital the patient has been dull, listless, and apathetic.

During the period when her urine was collected, she was entirely without spontaneous activity. The only reaction to questions was an occasional smile. Mild muscular resistance was present in the limbs, but no catalepsy.

20. A. G., age 52. Diagnosis: Depressive hallucinosis, recurrent attacks without any known toxic etiology.

The present attack has lasted about a month. During the time the urine was collected, patient was depressed and inactive; she sat quietly, rarely spoke or moved of her own accord, yet she was not retarded. Orientation clear.

21. J. O'D., age 26. Diagnosis: Dementia præcox. Duration, 4 months.

The patient has been mute and partially stuporous for two months; she shows practically no spontaneous activity; there is general muscular tension and the head springs forward if it is pushed backward; never moves unless strongly urged, and refuses to make any effort to care for herself.

22. M. E., age 39. Diagnosis: Manic-depressive insanity. 6 previous attacks; the present a manic attack; duration 6 weeks.

During the period of observation, the excitement had abated somewhat. She was still very talkative, but only mildly over-active with occasionally an episode of more intense excitement. At times she was irritable, threatening and scolding; she produced flights, rhymes and sound associations; distractibility well marked.

23. G. McG., age 21. Diagnosis: Excitement allied to manic-depressive insanity. Duration, 3 months.

At the time when the urine was collected, the excitement was subsiding somewhat. Previously she had shown considerable motor restlessness with heightened spirits and productivity in speech.

When under observation for the collection of urine, she showed less psycho-motor excitement, but there were frequent impulsive acts and destructive outbreaks; she still gave irrelevant or random replies, giggled excessively, and laughed boisterously.

24. E. G., age 38. Diagnosis: Manic-depressive insanity, (manic attack.) Duration 2 months. Four previous attacks. During the period when the urine was collected, the patient was in a state of mild exhilaration, accompanied by a moderate degree of busy activity and inclination to mischievous conduct; she produced a continuous stream of talk, characterized by quick shifting of topics, flights of ideas and sound associations.

25. D. R., age 76. Diagnosis: Manic-depressive insanity, Circular form. Attacks of excitement and depression, lasting from 2 to 4 months each, brief lucid periods at irregular intervals. The patient has remained in the hospital continuously for 31 years.

Her urine was collected while she was in a state of excitement. This was characterized by an exhilarated mood, a high degree of busy activity and a constant stream of talk, with flights of ideas and great distractibility.

26. Elizabeth Regan:

Admitted Dec. 16, 1905. Age 27. Dementia præcox. No improvement in four months. Akinetic.

27. Barbara Schmidt:

Admitted Jan. 2, 1906. Age 37. Infective exhaustive psychosis—constitutional peculiarity—gradual improvement—subsidence of hallucinations—retention of some probable constitutional traits. Akinetic.

28. Mary Straits:

Admitted Nov. 15, 1905. Age 46. Allied to manic-depressive insanity. A recurrent depression occurring during the involution age; the first attack at 42 followed by probable recovery. The second and present

attack being characterized by symptoms of both manic-depressive insanity and Involution Psychosis.—Gradual improvement. Akinetic, with agitation.

29. ANNIE MACHAUER:

Admitted Jan. 12, 1906. Age 28. Manic-depressive insanity. One previous attack 10 years ago which developed suddenly after an operation and was followed by recovery. Present attack an ill-defined depression developed after the death of her father; gradual improvement during the three months in which she has been in the hospital. Sudden improvement during the indol feeding. March 28, continued improvement, more cheerful expression, pleasant mood, movements natural, and fairly free. Patient had had nothing to say till the second day of indol feeding, when she came voluntarily and told freely of the diarrhœa from which she was suffering. It was found that she was well oriented.

30. LIZZIE LITTLE:

Admitted Feb. 22, 1906. Age 45. Alcoholic psychosis. No improvement during two months stay in the hospital. Apprehension and anxiety. Akinetic.

31. MARY MOHR:

Admitted Feb. 26, 1906. Age 36. Alcoholic psychosis. Constitutional peculiarities. Gradual improvement during two months stay in the hospital. Kinetic case.

32. MARY MCNULTY:

Readmitted Feb. 12, 1906. Age 35. Dementia præcox; duration 3 years; gradual decline. Akinetic. March 13, some improvement.

33. JENNIE PARKER:

Admitted March 6, 1906. Age 35. Manic-depressive insanity. Two previous attacks with recovery; present attack developed suddenly after childbirth; pronounced improvement in three weeks after admission. Kinetic.

34. ANNIE BRETT:

Admitted Mar. 14, 1906. Age 36. Dementia paralytica of at least 1½ years duration.

35. AMELIA BEHSMAN:

Admitted Dec. 5, 1905. Age 59. Alcoholic psychosis. Constitutional peculiarities. No permanent improvement during the 4 months stay in the hospital. No change during Indol feeding. Moderate diarrhœa. Frequent outbursts of temper and refractoriness. No movements.

36. JOSEPH CALLAHAN:

Admitted Jan. 10, 1906. Age 20. Allied to manic-depressive insanity. First attack—gradual onset after prolonged hard work; gradual improvement. No change during the indol feeding. Moderate diarrhœa.

37. ANNIE DIAMOND:

Admitted Sept. 23, 1905. Age 15. Allied to manic-depressive insanity—First attack, sudden development after prolonged hard work, and loss of sleep. Recovery in 5 months. No change during the indol feeding.

THE KINDS OF LACTIC ACID PRODUCED BY LACTIC ACID BACTERIA.

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(Received for publication, November 23, 1906.)

The writer has called attention to the similarity of the cultural and morphological characters of B. acidi lactici (Hueppe and others) with those of B. (lactis) aërogenes (Escherich), and also to the similarity of B. lactis acidi (Leichmann and others; Streptococcus lacticus, Kruse) and Streptococcus pyogenes.[1] In a later paper a resemblance is also shown to exist between Str. lacticus and Str. pyogenes[2] in respect to their behavior when inoculated into rabbits. Additional proof of the similarity of Str. lacticus with Str. pyogenes and of B. acidi lactici with B. aërogenes may now be presented.

The results of experiments of various authors on the kind of lactic acid formed in naturally soured milk do not agree. Günther and Thierfelder[3] state that the lactic acid present in naturally soured milk is not always solely the so-called fermentation lactic acid or, as it is also termed, the optically inactive or racemic variety.[4] They found that in most cases, to be sure "inactive acid" was present, but that often there was a preponderance of dextro-rotatory acid. The organism called B. lactis acidi (Streptococcus lacticus, Kruse) in pure culture produced invariably pure d-acid. Leichmann[5] found that B. lactis acidi in pure culture produced only d-acid. He expected

[1] *Journ. of Infect. Dis.*, iii, p. 173, 1906.

[2] *Ibid.*, iv, p. 87, 1907.

[3] *Arch. f. Hyg.*, xxv, p. 164, 1895.

[4] In accord with modern chemical nomenclature the expression "racemic acid" or "r-acid" will be employed in this paper in place of "inactive" or "i-acid."

[5] *Centralbl. f. Bakt. etc.*, ii, 2, p. 777, 1896.

to find the same acid in naturally soured milk, but observed that when milk soured at higher temperatures "inactive" acid was found. According to Thiele[1] there is present mostly d-acid at room temperature during the first few days, but after seven days "inactive" acid with some d-acid is present. At 37° C. d-acid is found after 36 hours, later "inactive," and finally "inactive mixed with l-acid." At variance with these results are those of Kozai.[2] This author found in milk, naturally soured at room temperature, pure d-acid or "d-acid mixed with a small amount of inactive acid," while at 37° he found "inactive with some l-acid." He concludes that bacteria forming l-acid grow preferably at the higher temperature. The presence of "inactive" acid is attributed to the activity of two different species of bacteria. Kozai also advances the idea that possibly one of the two acids is consumed by bacteria. Nef,[3] however, has observed the auto-transformation of active lactic acid to racemic acid. Utz[4] agrees with Kozai in noting that "inactive or inactive mixed with d-acid" are produced at room temperature, but could observe no difference at higher temperatures. MacKenzie[5] has criticised fundamentally the methods used by the above mentioned observers. He argues that, since active zinc lactates are soluble in 17.5 parts of water at 15° C., while inactive zinc lactate is soluble in 53 parts, there is the possibility, that in the process of crystallizing the difficultly soluble inactive salt any active salt present may remain in solution in the mother liquor. By repeated crystallization (the zinc salt being recrystallized in order to purify it) all traces of active salt may be lost sight of. The author concludes, therefore, that the results of Günther and Thierfelder, Kozai, Utz, and Thiele are not as contradictory as might appear on the surface, since their methods probably allowed some active salts to escape observation. MacKenzie also advances the view that racemic acid is never produced by the one species of micro-organism, but that separate species produce either one or the other of the two active acids.

[1] *Zeitschr. f. Hyg.*, xlvi, p. 394, 1904.
[2] *Ibid.*, xxxi, p. 337, 1899.
[3] *Ann. d. Chem.*, cccxxxv, pp. 243 and 290, 1904.
[4] *Centralbl. f. Bakt. etc.*, xi, 2, p. 600, 1903–04.
[5] *Journ. of the Chem. Soc.*, lxxxvii and lxxxviii, p. 373, 1905.

This opinion has been confirmed by carefully executed experiments with pure cultures of various moulds by MacKenzie and Harden.[1]

My attention was called to MacKenzie's paper after the greater part of the results presented in the accompanying tables had been gathered. Towards the end of my work a few tests of the mother liquor were made and the results fully bear out MacKenzie's criticism. In several instances inactive zinc salt crystallized leaving appreciable amounts of active salt in solution.

I have carried out two series of experiments. The object of the first series was to determine what kind of acid was produced by lactic acid bacteria in pure culture and in mixtures. The list included various strains of Str. lacticus, Str. pyogenes, Micr. lanceolatus, B. acidi lactici (B. aërogenes var. lacticus), and one strain of B. aërogenes and B. coli. Cultures of these organisms were added to "certified" milk, and control plates prepared to test the purity of the cultures. After the bulk of the cream had been removed,[2] the milk was sterilized for three days in the steam sterilizer and then preserved at room temperature for one week to test the sterility. The fermentation of the milk containing pure cultures was interrupted after three days; in the flasks with mixtures the fermentation was interrupted in three days in some instances, in others after five days. The results appear in Table I. All the streptococci produced d–acid. All the bacilli produced l–acid. In mixtures kept at 37° C. those which stood for three days contained d–acid, those which stood for five days r–acid. At room temperature pure d–acid is formed.

The second series of experiments was carried out in the following manner: Samples of milk from four different sources were examined. Each of the four kinds of milk was treated as follows. After removing the bulk of the cream of 21 one quart bottles the contents were emptied into a large container and thoroughly mixed, thus securing perfect uniformity of the milk.

[1] *Proc. of the Chem. Soc.*, xix, p. 48, 1903.

[2] By removing the cream but little fat remained to dissolve in the ether and the isolation of the lactic acid was facilitated. This was of particular importance, when the coagulum was granular or incomplete.

Plates in lactose litmus agar were then prepared from this fresh milk. The counts after three days were the smallest for "certified" milk, marked "C" in the table. The next higher count was found in milk "B," then follows "A," and "M" finally contained the largest number of bacteria.

TABLE I.

SPECIFIC ROTATORY POWER OF ZINC LACTATES PREPARED FROM LACTIC ACID, PRODUCED BY PURE CULTURES AND MIXTURES OF PURE CULTURES IN STERILIZED MILK.

Organism.	At 37° C.	Room Temperature.
Pneumococcus A.	−7.33	Not sufficient growth.
" B.	−7.33	"
Str. Pyogenes. (Mening.)	−7.14	"
" " (Tonsil.)	−7.81	"
" " (Scar. f.)	−7.37	"
" " "	−7.44	"
" lactic (Chic. milk)	−7.60	−7.49
" " " "	−7.29	−7.40
" " " "	−7.31	−8.00
" " " "	−7.20	−7.58
" " " "	−7.47	−7.38
" " " "	−7.24	−7.30
" " " "	−7.31	−7.49
" " " "	−7.14	−7.56
" " (Starter)	−7.20	−7.66
" " (Russell)	−7.21	−7.55
" " (N. Y. Sta.)	−7.16	−7.08
B. ac. lact. (Hueppe)	+7.30	+7.55
" " (Chic. milk)	+7.75	+7.74
" " (Lab. U. of C.)	+7.17	+7.30
B. aërog. " " " "	+7.11	+7.20
B. coli " " " "	+7.24	+7.61
B. ac. lact. + Str. lact.	−7.47	−7.31
" " " " "	−6.62	−7.61
" " " " "	inact.	−7.05
" " " " "	"	−7.06
" " " " pyog.	−5.32	+6.05
B. coli + Str. lact.	inact.	−7.06
B. aërog. + Str. lact.	−7.23	−7.33

Each one of these four lots of mixed milk was then transferred to 18 one-liter flasks, which had been sterilized. Nine flasks of each lot were kept at room temperature, the other nine at 37° C. One flask of each lot was removed every succeeding day and the zinc lactate prepared in the usual manner. The four kinds of milk were treated in the same fashion.

TABLE II.

SPECIFIC ROTATORY POWER OF ZINC LACTATES, PREPARED FROM LACTIC ACID, PRODUCED IN NATURALLY SOURED MILK.

Kind of Milk.	Days.	At 37° C.	At Room Temperature.
"C"	1	−7.59	−7.09
	2	−7.45	−7.19
	3	−7.34	−7.23
	4	−7.43	−7.43
	5	−7.31	−7.23
	6	−1.51	−7.53
	7	inact.	−7.38
	8	"	−7.79
	9	"	−7.30
	21	no test	−5.71
"A"	1	−7.34	−7.26
	2	−6.27	−7.76
	3	−4.86	−7.09
	4	−1.38	−7.11
	5	−0.90	−7.47
	6	inact.	−1.92
	7	"	inact.
	8	"	"
	9	+1.02	"
"B"	1	−7.14	−7.32
	2	−6.18	−7.18
	3	−4.07	−7.53
	4	−2.84	−5.70
	5	−1.11	−3.15
	6	inact.	−1.02
	7	"	inact.
	8	"	"
	9	+1.98	"
"M"	1	−7.34	−6.27
	2	−4.40	−5.31
	3	inact.	−4.80
	4	+0.76	−3.49
	5	+1.17	−2.03
	6	+1.43	+2.10
	7	+1.67	inact.
	8	+2.36	"
	9	+3.46	"

Examination of the figures in Table II shows that the first lot of milk, marked "C" and which was "certified" milk of the highest character, contained pure d-acid throughout five days at 37° C., on the sixth day a mixture of d-acid and l-acid, the former somewhat in excess. Finally, on the seventh, eighth,

and ninth days, r–acid only was present. At room temperature pure d–acid was present for nine days. It happened that one flask in excess of the regular number stood for 21 days, after which time a small amount of l–acid was found to be present.

The other three kinds of milk were market milk of good average quality. Milks "A" and "B": Both kinds show similar results. At 37° C., commencing on the first day both active acids were present in equal amounts so as to form r–acid. On the ninth day l–acid was slightly in excess. The mother liquor, however, retained increasing amounts of l–acid from the seventh day in "A," and l–acid was found in excess on the ninth day in "B." At room temperature d–acid was present for five and three days respectively, and r–acid on the seventh to ninth day. The mother liquor retained some d–acid in excess of r-acid for two days after the inactive zinc salt had been crystallized.

Milk "M": At 37° C. d–acid was formed on the first day, on the second there was d–acid with some l–acid, on the third r-acid, and after that the excess of l-acid increased steadily. At room temperature the increase of l–acid was much slower than at 37°C. and equalled the amount of d–acid on the seventh day without being in excess up to the ninth.

These experiments show that in milk from different sources different kinds of lactic acid may develop and that in "certified" milk the period during which d–acid predominates is longer than in ordinary milk. Preponderance of d–acid is also favored by preserving milk at low temperatures. Lactic acid in sour milk is produced chiefly by the activity of Streptococcus lacticus and Bacillus aërogenes. Since "certified" milk contains d–acid in excess of l–acid for a longer period of time both at 37° C. and at room temperature than the other kinds of milk, it seems safe to conclude that, from a bacteriological point of view, Str. lacticus predominates in approximate proportion to the purity of the milk.

Before the whey was separated from the coagulum and filtered for the purpose of isolating the lactic acid, the flask was thoroughly shaken and one cubic centimeter diluted 1:10,000 with sterile water and one cubic centimeter of the dilution plated in lactose litmus agar. After three days of incubation the colonies were counted. The results confirmed the conclusion

arrived at in a previous paper,[1] that the number of Str. lacticus increases with the progress of the acid fermentation, the number of B. aërogenes, however, decreases. This phenomenon was also noticed at 37° C., but was not so pronounced as at room temperature. Conn[2] has found 95 to 100 per cent. of all organisms in loppered milk to be of the B. lactis acidi type (Str. lacticus). It is to be observed, however, that this increase of Str. lacticus, which produces pure d–acid, is not accompanied by a relative increase of d–acid, on the contrary, l–acid increases in proportion to d–acid together with a decrease in numbers of B. aërogenes, although this latter organism produces pure l–acid. This apparent discrepancy is difficult to explain. We find frequent mention in the literature of the fact that micro-organisms prefer assimilating one of the two optical antipodes to the other, if the racemic variety is in the medium. The greater number of these observations show that d–acid is consumed and that l–acid is left intact or attacked but little. If this be true, we must assume according to Nef's theory of dissociation[3] that d–acid is dissociated to a higher degree than l–acid and is, therefore, consumed by some of the bacteria present as Kozai has supposed or, as Nef has suggested, that by auto-transformation depending on dissociation, d–acid is converted into l–acid respectively r–acid. This view receives support from the fact that at 37° C. the relative amount of l–acid increases more rapidly than at room temperature.

Another explanation is suggested on the basis of Buchner and Meisenheimer's[4] and Herzog's[5] discovery that lactose is converted into lactic acid by the activity of an endoenzyme produced by lactic acid bacteria. This enzyme may be liberated and diffused through the medium after the death of B. aërogenes and continue forming lactic acid from the lactose present. A minute amount of enzyme is all that is necessary to produce this result. Buchner and Meisenheimer did not test the rotatory power of the lactic acid produced by their enzyme, but state that it appeared

[1] *Loc. cit.*

[2] *Fifteenth Ann. Rep. Storrs Agric. Ex. Sta.*, 1903, p. 92.

[3] *Loc. cit.*

[4] *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 634, 1903.

[5] *Zeitschr. f. physiol. Chem.*, xxxvii, p. 381, 1903.

to be optically active. The greater increase of l-acid at 37° C. favors this view, as it is known that enzymes generally act more rapidly at temperatures higher than room temperature.

The opposing views of Leichmann, Günther, and Thierfelder on one side and of Kozai and others on the other can be harmonized by consideration of the following points:

1. It has been shown that in milk, obtained and preserved with aseptic precautions, d-acid is the chief product, both at room temperature and at 37° C. In milk of poorer quality the d-acid is rapidly neutralized by l-acid so that r-acid results and at incubator temperature the excess of d-acid is of such short duration that l-acid appears in equal amounts after forty-eight hours. Milk "M" in the table is of a quality above that of average market milk sold in bulk and it is quite in accord with the foregoing experiments to assume that milk with high bacterial content may contain r-acid in 24 hours at 37° C. In a later paper Günther and Thierfelder[1] have described experiments in which d-acid is formed chiefly at room temperature, but they found no constant variation at 37° C. The time of fermentation is not stated, but it is stated that the eight samples of milk examined were of unusually good quality. Nothing but d-acid could be expected under such conditions. The authors conclude that the kind of acid produced varies with different conditions and that they can find no adequate explanation of this phenomenon. The experiments discussed in this paper indicate that the quality of the milk examined influences the results, and the possibility of Kozai having worked with milk of a higher standard affords a reasonable explanation of the differences between his results and those of Günther and Thierfelder in their earlier work.

2. The time elapsing from the commencement of acid fermentation to the interruption has been shown to influence the results. Milk examined at an early period may contain d-acid, at a later period r-acid or even l-acid in excess. To obtain comparable results it is necessary to state the initial bacterial content, the length of time of fermentation, and the temperature at which the milk has been kept. Kozai and Thiele have examined milk at various stages of fermentation and have arrived at similar

[1] *Hyg. Rundschau*, x, p. 769, 1900.

results, viz., l–acid increases in relative amount to d–acid in approximate proportion to the period of fermentation.

It is also to be borne in mind that there are several factors which vitiate the accuracy of the results obtained by the usual method of preparing zinc lactates from sour milk. Evaporation of a solution of lactic acid or of lactates to concentration causes the formation of lactic anhydride which is lævo-rotatory and may change the specific rotation from right to left. The salts of this anhydride are highly rotatory but in the opposite direction.[1] Another source of error is the point mentioned above to which MacKenzie has called attention. If any active salt is present in a solution of inactive, the former will remain in the mother liquor when the latter crystallizes. By repeated crystallization the last traces of active salt will then be washed away and lost sight of. To what extent these sources of error may have influenced the results of the authors mentioned it is difficult to estimate. The comparability of results is probably not affected to a high degree, but it is more than likely that active salts have been frequently overlooked.

Summarizing the results of the experiments described we find that:

1. Milk naturally soured at room temperature contains chiefly d–acid. Milk soured at 37° C. contains chiefly r–acid with l–acid in excess if allowed to stand several days.

2. Streptococcus lacticus and Str. pyogenes produce the same kind of lactic acid, *i. e.* d–acid. B. aërogenes from milk (B. acidi lactici) and the ordinary laboratory strain of B. (lactis) aërogenes (Escherich) produce the same kind of lactic acid, *i. e.* l–acid.

3. The lactic acid produced in naturally soured milk varies:

(a.) According to the relative numbers of Str. lacticus and B. aërogenes present. The higher the number of B. aërogenes the more l–acid is produced.

(b.) According to the temperature at which the fermentation takes place, other conditions being equal. At 37° C. relatively more l–acid is formed than at room temperature.

[1] Jungfleisch and Godchot, *Compt. rend. de l'Acad. des sci.*, cxlii, p. 515, 1906.

(c.) According to the length of time the fermentation has lasted. The longer the time the more l-acid is formed.

4. In "certified" milk, d-acid only was present at room temperature for nine days, while both d-acid and l-acid were present in milk of poorer quality after one to four days. At 37° l-acid was apparent after six days in "certified" milk and on the second day in other milk. It seems as if the purer the milk the longer the excess of d-acid persists.

5. Racemic lactic acid is the result of the formation of pure d-acid and pure l-acid by at least two different species of micro-organisms. Racemic lactic acid is not known to be the product of one species only.

6. Since it is known that B. aërogenes forms other acids besides lactic acid, often in appreciable amounts, while Str. lacticus produces almost pure d-acid, the presence of d-acid may be taken as indicating desirable conditions for dairy work, because this shows the absence of the fermentation products of B. aërogenes, *i. e.* volatile acids, gas, and ethyl alcohol.

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